

Microbial Fermentation Method for Production of N-Acetyl-D-Glucosamine and/or D-Glucosamine Salt

Field of Technology

5 This invention belongs to the field of microbial fermentation. Specifically, this invention relates with production of N-Acetyl-D-Glucosamine by microbial fermentation, and further preparation method of D-Glucosamine Salt.

Background Technology

10 N-Acetyl-D-Glucosamine (NAG or GlcNAc), also called N-Acetyl-Glucosamine or N-AcetylGlucosamine, is a basic constituent unit for multiple important polysaccharides in biological cells, and has an important physiological function in organisms. N-Acetyl-D-Glucosamine may be used clinically to: Increase the function of human immune system; inhibit growth of malignancy or fibrocytes;
15 treatment effectively various inflammations; and be as a low-heat sweetener in diabetic patients, and a food additive for infants and young children; and so on. Hydrolysis of N-Acetyl-D-Glucosamine may be used to produce D-Glucosamine Hydrochloride, which may be used as a food additive for fighting and preventing cancer, and lowering blood lipids and blood pressure, and is presently a
20 third-generation food additive with healthcare function in chitin health food series. Moreover, N-Acetyl-D-Glucosamine is a major raw material for synthesis of Chlorozotocin, an anticancer agent; as a biochemical reagent, it may also be used as an immune adjuvant for resisting bacterial infection, and an activating agent for counteracting influenza viruses in human body.

25 Now all over the world, a lot of patients suffer from arthritis pain to different extents. In the United States only, there are 33 millions of patients suffering from arthritis and arthralgia; in our country, more than more than 0.15 billions of patients suffering arthritis and arthralgia. As D-Glucosamine products have special therapeutic and healthcare effects against arthritis and arthralgia, it has been used
30 extensively, and now has become a very important raw material in domestic and foreign markets.

It is believed that N-Acetyl-D-Glucosamine has similar effects to D-Glucosamine. It is know that intake of N-Acetyl-D-Glucosamine can induce

production of new cartilage and block episodes of osteoarthritis, or is used to treat osteoarthritis in some cases. As D-Glucosamine is bitter while N-acetyl-D-Glucosamine is 50% sweet of sucrose and is easily ingested. Therefore, N-acetyl-D-Glucosamine has aroused concern as alternative of D-Glucosamine.

5 Presently, Glucosamine is mainly derived from biological extract at home and abroad. Biological extraction is mainly to extract chitin or chitosan from shrimp and crab shells and then hydrolyzed by concentrated hydrochloric acid, or extract from citrate slags by acid or alkali. The annual output is approximately 20,000 tons. However, when extracted from shrimp and crab shells, each ton of the product is
10 accompanied with a great amount of waste residue, and more than 100 tons of waste water; when extracted from citrate residue, each ton of the product is accompanied with 30-50 tons of waste acid residue - the process is a high-pollution process and has been prohi-
15 gited in many places. Moreover, Glucosamine extracted from aquatic shells is not suitable for patients sensitive to aquatic products in whom it may result in severe allergy problems or even life-threatening. Besides, the biological extraction and purification processes are complicated, and the product smells fishy and is unstable. Moreover, due to environmental pollution, it is inevitable that Glucosamine extracted from shrimp and crab shells may be contaminated by heavy metals.

 Therefore, Glucosamine produced by biological extraction method cannot meet
20 the needs of people in quantity and quality; it has to develop a new alternative method. If it is produced by chemical synthesis method, there are the following three shortcomings: High production cost; severe environmental pollution; and potential safety risks. Now the method has been cancelled at home and abroad. In comparison, the microbial fermentation method for production of Glucosamine is a good route, in
25 which, glucose and inorganic salts are as raw materials, and undergo liquid fermentation by excellent strains, and separation, concentration, and purification to directly product Glucosamine. No noxious gas is produced during production. Glucosamine produced by fermentation method does not smell fishy, and the production resources are not limited. As well, strain improvement is carried out by
30 metabolic engineering, with high output, and great potential for large-scale industrial production. Therefore, the microbial fermentation method for production of Glucosamine has a significant revolution to replace conventional biological extraction, and has an advantage in the cost and makes a contribution to

environmental protection in reducing pollution of three wastes.

The routine methods of microbial fermentation for production of N-Acetyl-D-Glucosamine include: Method (for example, US5998173, "Process for producing N-acetyl-D-glucosamine") to produce N-Acetyl-D-Glucosamine from chitin manufactured from shrimp shells by enzymatic degradation; the enzymes are produced microbially; Method (for example, US20030073666A1, "N-acetyl-D-glucosamine and process for production of N-acetyl-D-glucosamine") to produce N-Acetyl-D-Glucosamine from chitin manufactured and purified from fungal residue (such as fungal residue of *Aspergillus niger*, used by citrate fermentation) by enzymatic degradation or acid-partial hydrolysis; the enzymes are produced microbially (by *Trichoderma*); method (for example, US20110059489A1, "Method for fermentative production of N-acetyl-D-glucosamine by microorganism") to produce N-Acetyl-D-Glucosamine by fermentation of *Trichoderma*, using directly glucose as carbon source, other than chitin and chitosan oligosaccharide produced from fungal residue or shrimp shells; method (for example, JP2004283144A, "Method for producing glucosamine and N-acetylglucosamine") to produce N-Acetyl-D-Glucosamine from incubation of *Chlorella* cells infected with *Chlorovirus*, or of recombinant *Escherichia coli* introduced with the gene of *Chlorovirus*; method (for example, US6,372,457, "Process and materials for production of glucosamine"; WO2004/003175, "Process and materials for production of glucosamine and N-acetylglucosamine") to produce D-Glucosamine or N-Acetyl-D-Glucosamine by fermentation, using genetically modified microorganism, particularly genetically modified *Escherichia coli*.

The method for production of N-acetyl-D-glucosamine from degradation of chitin derived from the shells of shellfish such as crab and shrimp, by microorganism or by enzymes produced microbially, is relatively conventional, and has the problems of low yield, high cost, and insufficient animal source, etc. The method for production of N-acetyl-D-glucosamine from incubation of *Chlorella* cells infected with *Chlorovirus* is of complicated operation, etc., as it is involved with the step of crushing cells to obtain N-acetyl-D-glucosamine. The method for production of N-acetyl-D-glucosamine by *Trichoderma*, using glucose as carbon source, had the advantages of requiring no chitin or chitin oligosaccharide as carbon source, produced from the shells of shellfish or from fungi dregs. However, as the fungi such

as *Trichoderma* are of low fermentation temperature (27°C), long term (10 days), and relatively low yield (15 mg/mL), and thus are of long production cycle, high cost, and easy contamination, seriously restricting industrial application of the method.

Clearly, aiming at the increasing market demand for Glucosamine, the method to produce N-Acetyl-D-Glucosamine by genetically modified microorganism is an important method with application potential to realize large-scale industrialization. New, genetically modified microorganism may be obtained by multiple modes, such as gene recombination, gene transfer, gene mutation, gene deletion, gene overexpression, or change to metabolic pathway, etc.

US6,372,457, a United State patent, discloses the method and materials to produce D-Glucosamine by microbial fermentation. This Invention is involved with genetically modified microorganism for the method for production Glucosamine, as well as recombinant nucleic acid molecules, and proteins produced by the said recombinant nucleic acid molecules. The said genetically modified microorganism in this Invention are mainly involved with the genetic modification increasing the activities of Glucosamine-6-Phosphate Synthase, including multiple gene mutations or amino acid deletion and substitution. However, the patient is not involved with changes of Glucosamine-6-Phosphate Synthase gene promoter replacement or deletion, leading to decreased or increased activities of Glucosamine-6-Phosphate Synthase. Moreover, the patient is mainly intended to produce D-Glucosamine, the only one target product, by genetic modification of Glucosamine-6-Phosphate Synthase, and is not involved with production of N-Acetyl-D-Glucosamine. Furthermore, as D-Glucosamine is quite unstable in fermentation broth, degradation products may be possibly toxic to microorganism. This production mode of D-Glucosamine by genetic modification is of very low yield, and is restricted in practical application.

The biosynthetic method for production of D-Glucosamine and N-Acetyl-D-Glucosamine is disclosed in WO2004/003175. The method is to produce Glucosamine and/or N-Acetyl-D-Glucosamine by fermentation of genetically modified microorganism. This Invention also discloses the genetically modified microorganism for production of Glucosamine and N-Acetyl-D-Glucosamine. Additionally, this Invention also describes the recovery method of N-Acetyl-D-Glucosamine produced by fermentation method, including method for

production of high-purity N-Acetyl-D-Glucosamine. This Invention also discloses the method for production of D-Glucosamine from N-Acetyl-D-Glucosamine. The said genetically modified microorganism in this Invention are mainly involved with the genetic modification increasing the activities of Glucosamine-6-Phosphate Acetyltransferase. It has been reported and demonstrated in previous literatures that the expression of yeast Glucosamine-6-Phosphate Acetyltransferase gene (GNA1) in *Escherichia coli* may acetylate Glucosamine-6-Phosphate into Acetylglucosamine-6-Phosphate (Mio T1, Yamada-Okabe T, Arisawa M, Yamada-Okabe H: *Saccharomyces cerevisiae* GNA1, an essential gene encoding a novel acetyltransferase involved in UDP-N-acetylglucosamine synthesis, *J Biol Chem.*, 1999 Jan 1;274(1):424-9).

In production of N-Acetyl-Glucosamine, as the microorganism for high-density fermentation requires a large amount of oxygen; it requires persistent stirring and thus increases energy consumption. Moreover, persistent stirring may produce a lot of forms, thus influencing the output.

Contents of the Invention

In this Invention, the microorganism is reformed by genetic modification, so that the microorganism may produce N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt in high efficiency and high output, thus to decrease production cost for industrial scale production.

Specifically speaking in this Invention, by expression of vitreoscilla hemoglobin (Vhb) in microorganism, it may increase the microorganism's utilization ability for dissolved oxygen, accelerate synthesis of proteins and metabolic products, promote microbial growth, increase fermentation potency and level, so that the microorganism may produce N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt in higher efficiency and higher product under limited oxygen conditions.

On top of the above contents, this Invention is further involved with one or more of the following contents:

1. By increasing the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganisms, this Invention may strengthen phosphorylation of N-Acetyl-D-Mannosamine (ManNAc) into N-Acetyl-D-Mannosamine-6-Phosphate (ManNAc-6-P) in microorganism, so that microorganism may produce more

efficiently N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine in a higher yield.

2. By increasing the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, strengthen transformation of N-Acetyl-D-Mannosamine-6-Phosphate (ManNAc-6-P) in microorganism into N-Acetyl-D-Glucosamine-6-Phosphate (GlcNAc-6-P), excreted into extracellular domain to become N-Acetyl-D-Glucosamine (GlcNAc), so that microorganism may produce more efficiently N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt in a higher yield.

3. By increasing the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and meanwhile decreasing preferably the effects of Glucosamine-6-Phosphate Synthase (GlmS, also called L-Glutamine-D-fructose-6-Phosphate Aminotransferase), strengthen amination of Glucose-6-Phosphate (Glc-6-P) in microorganism into D-Glucosamine-6-Phosphate (GlcN-6-P). The reaction catalyzed by D-Glucosamine-6-Phosphate Deaminase (NagB) is reversible, while that catalyzed by Glucosamine-6-Phosphate Synthase (GlmS) is irreversible, but has a severe problem for product inhibition. When the NagB-catalyzed reaction proceeds in the direction from Glc-6-P to produce GlcN-6-P, its function is the same as that of GlmS, and may replace GlmS, without any problem for product inhibition. Increase effects of NagB, accelerate the NagB-catalyzed reaction to proceed in the direction from Glc-6-P to GlcN-6-P, meanwhile decrease preferably the effects of GlmS, mitigate the product inhibition problem of GlmS, to achieve the purpose of increasing GlcN-6-P, so that such microorganism may produce more efficiently N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt in a higher yield.

4. By increasing the effects of Glucosamine-6-Phosphate Synthase (GlmS, also called L-Glutamine-D-Fructose-6-Phosphate Aminotransferase) in microorganism, and meanwhile decreasing the effects of D-Glucosamine-6-Phosphate Deaminase (NagB), strengthen amination of Glucose-6-Phosphate (Glc-6-P) in microorganism into D-Glucosamine-6-Phosphate (GlcN-6-P). The reaction catalyzed by D-Glucosamine-6-Phosphate Deaminase (NagB) is reversible. When the NagB-catalyzed reaction proceeds in the direction from Glc-6-P to produce GlcN-6-P, its function is opposite to that of GlmS, and may offset the effects of GlmS. Decreasing the effects of NagB, block the NagB-catalyzed reaction to proceed in the direction

from GlcN-6-P to Glc-6-P, and meanwhile overexpress GlmS, accelerate GlmS-catalyzed amination of Glc-6-P into GlcN-6-P, to achieve the purpose of increasing GlcN-6-P, so that microorganism may produce more efficiently N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt in a higher yield.

5 5. By increasing the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB), strengthen transformation of UDP-N-Acetyl-D-Glucosamine (UDP-GlcNAc) in microorganism into N-Acetyl-D-Mannosamine (ManNAc), so that microorganism may produce more efficiently N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt in a higher yield.

10 6. Decrease the effects of reuptake of target products into cells, or of relevant enzymes or proteins to degrade beneficial intermediates, increase sugar conversion rate and N-Acetyl-D-Glucosamine yield in microorganism, so that microorganism may produce more efficiently N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt in a higher yield. Include but are not limited to one or more of
15 the following contents:

(1) Decrease the effects of Mannose transporter EIIM, P/III^{man} (ManXYZ) in microorganism, and block return of hexoses such as N-Acetyl-D-Glucosamine (GlcNAc) to cells for degradation.

20 (2) Decrease the effects of N-Acetylneuraminate Lyase (NanA) in microorganism, and block degradation of N-Acetyl-D-Mannosamine (ManNAc) in microorganism.

25 (3) Decrease the effects of N-Acetyl-D-Glucosamine-6-Phosphate Deacetylase (NagA) in microorganism, block transformation of N-Acetyl- D- Glucosamine- 6- Phosphate (GlcNAc-6- P) into D-Glucosamine-6-Phosphate (GlcN-6-P) in microorganism.

(4) Decrease the effects of N-Acetyl-D-Glucosamine Specific Enzyme II^{Nag} (NagE), and block transportation of N-Acetyl-D-Glucosamine (GlcNAc) into microbial cells for degradation.

30 (5) By increasing the effects of PhosphoGlucosamine Mutase (GlmM) in microorganism, strengthen transformation of D-Glucosamine-6-Phosphate (GlcN-6-P) into D-Glucosamine-1-Phosphate (GlcN-1-P).

(6) by increasing the effects of bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (also called Glucosamine-1-Phosphate Acetyl Transferase, GlmU),

strengthen transformation of D-Glucosamine-1-Phosphate (GlcN-1-P) into N-Acetyl-D-Glucosamine-1-Phosphate (GlcNAc-1-P), and further transformation into UDP-N-Acetyl-D-Glucosamine (UDP-GlcNAc).

Based on one implementation scheme of this Invention, this Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb); and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

Preferably further include C) Deacetylation of N-Acetyl-D-Glucosamine (GlcNAc) to produce D-Glucosamine Salt.

In this Invention, the microorganism contains at least one transformation of recombinant nucleic acid molecules of the nucleotide sequence encoding vitreoscilla hemoglobin (Vhb).

In this Invention, using vitreoscilla hemoglobin (Vhb) as object, random base replacement is introduced by error-prone PCR random mutagenesis method for DNA reorganization, to screen an ideal mutant of the protein. The random mutagenesis method for introducing mutations by error-prone PCR is to screen an ideal mutant by introducing random base replacement, while DNA reorganization may increase more significantly the probability for benign mutations, thus to obtain mutants with higher application value. To increase activities of vitreoscilla hemoglobin (Vhb), the microorganism is rebuilt by the combination of error-prone PCR and DNA reorganization, and the mutant gene is controlled by promoter for oxygen regulation to carry out expression and screening, to obtain mutant protein with higher activities than wild type under limited oxygen conditions.

On the other hand, the nucleotide sequence encoding vitreoscilla hemoglobin (Vhb) contains at least one genetic modification that may increase the activities of vitreoscilla hemoglobin (Vhb). Preferably, the said genetic modification contains one or more substitutions at the following corresponding sites of the amino acid sequence SEQ ID NO:61: Methionine at Site 45 is substituted by leucine, cysteine at Site 86 is substituted by glycine, and tyrosine at Site 95 is substituted by serine. More

preferably, the nucleic acid sequence encoding the said vitreoscilla hemoglobin (Vhb) is SEQ ID NO: 64; the amino acid sequence of the said vitreoscilla hemoglobin (Vhb) is SEQ ID NO:65.

On the other hand, at least approximately 30% (more preferably at least approximately 50%, further more preferably at least approximately 70%, further more preferably at least approximately 80%, and further more at least approximately 90%, and most preferably at least approximately 95%) of the amino acid sequence of the said vitreoscilla hemoglobin (Vhb) is the same as that of SEQ ID NO:61, where the said vitreoscilla hemoglobin (Vhb) is active.

On the other hand, the said vitreoscilla hemoglobin (Vhb) has the amino acid sequence of SEQ ID NO:61.

On the other hand, the gene copies in the recombinant nucleic acid molecules, encoding vitreoscilla hemoglobin (Vhb), are more than or equal to 1.

On the other hand, the recombinant nucleic acid molecules contain an endogenous promoter, with a higher expression level of promoters, enhancers, and fusion sequences, etc., than those of natural endogenous promoters. Preferably, the recombinant nucleic acid molecules contains a higher expression level of promoters than that of natural endogenous promoters, such as HCE promoter, gap promoter, trc promoter, and T7 promoter, etc.; more preferably, the recombinant nucleic acid molecules contain trc promoter. trc promoter is a combined promoter of trp promoter and lac promoter, with a higher transcription efficiency than that of trp, and with strong promoter characteristics to be regulated by lacI repressor protein.

In this Invention, microorganism transformed by recombinant nucleic acid molecules transform are selected from free type (that is to say, recombinant nucleic acid molecules are mounted into plasmids) and integrated type (that is to say, recombinant nucleic acid molecules are integrated into the genomes of microorganism). Preferably, recombinant nucleic acid molecules are integrated into the genomes of microorganism.

Based on the preferably optimized implementation scheme, the said microorganism contains one or more of the following genetic modifications:

(1) Contain at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism;

(2) Contain at least one genetic modification that may increase the effects of

N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism;

(3) Contain at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and preferably contain at least one genetic modification that may decrease the effects of

5 Glucosamine-6-Phosphate Synthase (GlmS);

(4) Contain at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, and preferably contain at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NagB)

10 (5) Contain at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism;

In respect to the above (1), the genetic modification to increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism is selected from: a) increasing effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism; and/or b) overexpression of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism.

A technician in this field may understand that, to increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, it may be realized by screening for a gene mutant of N-Acetyl-D-Mannosamine Kinase (NanK), encoding N-Acetyl-D-Mannosamine Kinase (NanK) with increased activities. The screening for a NanK gene mutant may be achieved by error-prone PCR technology to produce a high-frequency mutant gene. To increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, it may also be realized by increasing its gene copies or replacing it with a promoter with a higher expression level than that of the natural promoter, for overexpression of N-Acetyl-D-Mannosamine Kinase (NanK). In a specific implementation scheme, the microorganism is transformed by molecules of at least recombinant nucleic acid, containing at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism.

30 In a preferably optimized implementation scheme, the microorganism is transformed by molecules of at least recombinant nucleic acid, containing one nucleotide sequence encoding N-Acetyl-D-Mannosamine Kinase (NanK).

On the other hand, the nucleotide sequence encoding N-Acetyl-D-Mannosamine

Kinase (NanK) contains at least one genetic modification that may increase the activities of N-Acetyl-D-Mannosamine Kinase (NanK). Preferably the said genetic modification contains one or more substitutions at the following corresponding sites of SEQ ID NO: 17: Substitution of lysine at site 36 by arginine, substitution of
5 isoleucine at Site 103 by methionine, and substitution of arginine at Site 223 by serine. More preferably, the nucleic acid sequence encoding the said N-Acetyl-D-Mannosamine Kinase (NanK) is SEQ ID NO: 26; the amino acid sequence of the said N-Acetyl-D-Mannosamine Kinase (NanK) is SEQ ID NO: 27.

On the other hand, at least approximately 30% (more preferably at least
10 approximately 50%, further more preferably at least approximately 70%, further more preferably at least approximately 80%, and further more at least approximately 90%, and most preferably at least approximately 95%) of the amino acid sequence of the said N-Acetyl-D-Mannosamine Kinase (NanK) is the same as that of SEQ ID NO: 17, where the said N-Acetyl-D-Mannosamine Kinase (NanK) is active.

On the other hand, the said N-Acetyl-D-Mannosamine Kinase (NanK) has the
15 amino acid sequence of SEQ ID NO: 17.

On the other hand, the gene copies in the recombinant nucleic acid molecules, encoding N-Acetyl-D-Mannosamine Kinase (NanK), are increased.

On the other hand, the recombinant nucleic acid molecules contain an
20 endogenous promoter, with a higher expression level of promoters, enhancers, and fusion sequences, etc., than those of natural endogenous promoters. Preferably, the recombinant nucleic acid molecules contains a higher expression level of promoters than that of natural endogenous promoters, such as HCE promoter, gap promoter, trc promoter, and T7 promoter, etc.; more preferably, the recombinant nucleic acid
25 molecules contain trc promoter. trc promoter is a combined promoter of trp promoter and lac promoter, with a higher transcription efficiency than that of trp, and with strong promoter characteristics to be regulated by lacI repressor protein.

In this Invention, microorganism transformed by recombinant nucleic acid molecules transform are selected from free type (that is to say, recombinant nucleic
30 acid molecules are mounted into plasmids) and integrated type (that is to say, recombinant nucleic acid molecules are integrated into the genomes of microorganism). Preferably, recombinant nucleic acid molecules are integrated into the genomes of microorganism.

In another preferably optimized implementation scheme, the microorganism contains at least one genetic modification of the natural endogenous promoter to the gene encoding N-Acetyl-D-Mannosamine Kinase (NanK). Preferably, the natural endogenous promoter with the gene encoding N-Acetyl-D-Mannosamine Kinase (NanK) is replaced by a promoter with a higher expression level, such as HCE promoter, gap promoter, trc promoter, or T7 promoter, etc.; more preferably, the natural endogenous promoter with the gene encoding N-Acetyl-D-Mannosamine Kinase (NanK) is replaced by trc promoter.

In respect to the above (2), the genetic modification increasing the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism is selected from a) increasing the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism; and/or b) overexpression of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism.

A technician in this field may understand that, to increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, it may be realized by screening for a gene mutant of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE), encoding N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) with increased activities. The screening for a NanE gene mutant may be achieved by error-prone PCR technology to produce a high-frequency mutant gene. To increase the effects of N-Acetyl-D-Mannosamine-Phosphate Epimerase (NanE) in microorganism, it may also be realized by increasing its gene copies or replacing it with a promoter with a higher expression level than that of the natural promoter, for overexpression of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE). In a specific implementation scheme, the microorganism is transformed by molecules of at least recombinant nucleic acid molecules, containing at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism.

In a preferably optimized implementation scheme, the microorganism is transformed by molecules of recombinant nucleic acid, containing one nucleotide sequence encoding N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE).

On the other hand, the nucleotide sequence encoding N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) contains at least one genetic modification that may increase the activities of N-Acetyl-D-Mannosamine-

6-Phosphate Epimerase (NanE). Preferably the said genetic modification contains one or more substitutions at the following corresponding sites of SEQ ID NO: 29: Substitution of cysteine at Site 133 by arginine, and substitution of tyrosine at Site 187 by histidine. More preferably, the nucleic acid sequence encoding N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) is SEQ ID NO: 56; the amino acid sequence of the said N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) is SEQ ID NO: 57.

On the other hand, at least approximately 30% (more preferably at least approximately 50%, further more preferably at least approximately 70%, further more preferably at least approximately 80%, and further more at least approximately 90%, and most preferably at least approximately 95%) of the amino acid sequence of the said N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) is the same as that of SEQ ID NO: 29, where the said N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) is active.

On the other hand, the said N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) has the amino acid sequence of SEQ ID NO: 29.

On the other hand, the gene copies in the recombinant nucleic acid molecules, encoding N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE), are increased.

On the other hand, the recombinant nucleic acid molecules contain an endogenous promoter, with a higher expression level of promoters, enhancers, and fusion sequences, etc., than those of natural endogenous promoters. Preferably, the recombinant nucleic acid molecules contains a higher expression level of promoters than that of natural endogenous promoters, such as HCE promoter, gap promoter, trc promoter, and T7 promoter, etc.; more preferably, the recombinant nucleic acid molecules contain trc promoter.

In this Invention, microorganism transformed by recombinant nucleic acid molecules transform are selected from free type (that is to say, recombinant nucleic acid molecules are mounted into plasmids) and integrated type (that is to say, recombinant nucleic acid molecules are integrated into the genomes of microorganism). Preferably, recombinant nucleic acid molecules are integrated into the genomes of microorganism.

In another preferably optimized implementation scheme, the microorganism contains at least one genetic modification of the natural endogenous promoter to the

gene encoding N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE). Preferably, the natural endogenous promoter with the gene encoding N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) is replaced by a promoter with a higher expression level, such as HCE promoter, gap promoter, trc promoter, or T7 promoter, etc.; more preferably, the natural endogenous promoter with the gene encoding N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) is replaced by trc promoter.

In respect to the above (3), the genetic modification increasing the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism is selected from a) increasing the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism; and/or b) overexpression of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism.

A technician in this field may understand that, to increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, it may be realized by screening for a gene mutant of D-Glucosamine-6-Phosphate Deaminase (NagB), encoding D-Glucosamine-6-Phosphate Deaminase (NagB) with increased activities. The screening for a NanG gene mutant may be achieved by error-prone PCR technology to produce a high-frequency mutant gene. To increase the effects of D-Glucosamine-6-Phosphate Deaminase (NanB) in microorganism, it may also be realized by increasing its gene copies or replacing it with a promoter with a higher expression level than that of the natural promoter, for overexpression of D-Glucosamine-6-Phosphate Deaminase (NanB). In a specific implementation scheme, the microorganism is transformed by molecules of at least one recombinant nucleic acid, containing at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NanB) in microorganism.

In a preferably optimized implementation scheme, the microorganism is transformed by molecules of at least recombinant nucleic acid, containing one nucleotide sequence encoding D-Glucosamine-6-Phosphate Deaminase (NanB).

On the other hand, the nucleotide sequence encoding D-Glucosamine-6-Phosphate Deaminase (NanB) contains at least one genetic modification that may increase the activities of D-Glucosamine-6-Phosphate Deaminase (NanB).

On the other hand, the gene copies in the recombinant nucleic acid molecules,

encoding D-Glucosamine-6-Phosphate Deaminase (NanB), are increased.

On the other hand, the recombinant nucleic acid molecules contain an endogenous promoter, with a higher expression level of promoters, enhancers, and fusion sequences, etc., than those of natural endogenous promoters. Preferably, the recombinant nucleic acid molecules contains a higher expression level of promoters than that of natural endogenous promoters, such as HCE promoter, gap promoter, trc promoter, and T7 promoter, etc.; more preferably, the recombinant nucleic acid molecules contain trc promoter.

In this Invention, microorganism transformed by recombinant nucleic acid molecules transform are selected from free type (that is to say, recombinant nucleic acid molecules are mounted into plasmids) and integrated type (that is to say, recombinant nucleic acid molecules are integrated into the genomes of microorganism). Preferably, recombinant nucleic acid molecules are integrated into the genomes of microorganism.

In another preferably optimized implementation scheme, the microorganism contains at least one genetic modification of the natural endogenous promoter to the gene encoding D-Glucosamine-6-Phosphate Deaminase (NanB). Preferably, the natural endogenous promoter with the gene encoding D-Glucosamine-6-Phosphate Deaminase (NanB) is replaced by a promoter with a higher expression level, such as HCE promoter, gap promoter, trc promoter, or T7 promoter, etc.; more preferably, the natural endogenous promoter with the gene encoding D-Glucosamine-6-Phosphate Deaminase (NanB) is replaced by trc promoter.

In this Invention, the genetic modifications decreasing the effects of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism is selected from a) decreasing effects of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism; and/or b) reducing expression of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, including but not limited to: Partial or complete deletion, or partial or complete deactivation of the endogenous gene encoding Glucosamine-6-Phosphate Synthase (GlmS), and/or partial or complete deletion, or partial or complete deactivation of the natural endogenous promoter with the gene encoding Glucosamine-6-Phosphate Synthase (GlmS). Preferably, the genetic modification decreasing the effects of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism is complete deletion (loss) of the natural endogenous promoter with

the gene encoding Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

In a specific implementation scheme, the microorganism is transformed by molecules of at least one recombinant nucleic acid, containing at least one genetic modification that may decrease the effects of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

In respect to the above (4), the genetic modification increasing the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism is selected from a) increasing the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism; and/or b) overexpression of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

A technician in this field may understand that, to increase the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, it may be realized by screening for a gene mutant of D-Glucosamine-6-Phosphate Deaminase (NagB), encoding D-Glucosamine-6-Phosphate Deaminase (NagB) with increased activities. The screening for a GlmS gene mutant may be achieved by error-prone PCR technology to produce a high-frequency mutant gene. To increase the effects of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, it may also be realized by increasing its gene copies or replacing it with a promoter with a higher expression level than that of the natural promoter, for overexpression of Glucosamine-6-Phosphate Synthase (GlmS). In a specific implementation scheme, the microorganism is transformed by molecules of at least one recombinant nucleic acid, containing at least one genetic modification that may increase the effects of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

In a preferably optimized implementation scheme, the microorganism is transformed by molecules of at least recombinant nucleic acid, containing one nucleotide sequence encoding Glucosamine-6-Phosphate Synthase (GlmS).

On the other hand, the nucleotide sequence encoding Glucosamine-6-Phosphate Synthase (GlmS) contains at least one genetic modification that may increase the activities of D-Glucosamine-6-Phosphate Synthase (GlmS).

On the other hand, the gene copies in the recombinant nucleic acid molecules, encoding D-Glucosamine-6-Phosphate Synthase (GlmS), are increased.

On the other hand, the recombinant nucleic acid molecules contain an endogenous promoter, with a higher expression level of promoters, enhancers, and

fusion sequences, etc., than those of natural endogenous promoters. Preferably, the recombinant nucleic acid molecules contains a higher expression level of promoters than that of natural endogenous promoters, such as HCE promoter, gap promoter, trc promoter, and T7 promoter, etc.; more preferably, the recombinant nucleic acid molecules contain trc promoter.

In this Invention, microorganism transformed by recombinant nucleic acid molecules transform are selected from free type (that is to say, recombinant nucleic acid molecules are mounted into plasmids) and integrated type (that is to say, recombinant nucleic acid molecules are integrated into the genomes of microorganism). Preferably, recombinant nucleic acid molecules are integrated into the genomes of microorganism.

In another preferably optimized implementation scheme, the microorganism contains at least one genetic modification of the natural endogenous promoter to the gene encoding Glucosamine-6-Phosphate Synthase (GlmS). Preferably, the natural endogenous promoter with the gene encoding Glucosamine-6-Phosphate Synthase (GlmS) is replaced by a promoter with a higher expression level, such as HCE promoter, gap promoter, trc promoter, or T7 promoter, etc.; more preferably, the natural endogenous promoter with the gene encoding Glucosamine-6-Phosphate Synthase (GlmS) is replaced by trc promoter.

In this Invention, the genetic modifications decreasing the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism are selected from a) decreasing effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism; and/or b) reducing expression of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, including but not limited to: Partial or complete deletion, or partial or complete deactivation of the endogenous gene encoding D-Glucosamine-6-Phosphate Deaminase (NagB), and/or partial or complete deletion, or partial or complete deactivation of the natural endogenous promoter with the gene encoding Glucosamine-6-Phosphate Deaminase (NagB). Preferably, the genetic modification decreasing the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism is complete deletion (loss) of the natural endogenous promoter with the gene encoding D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism.

In a specific implementation scheme, the microorganism is transformed by

molecules of at least one recombinant nucleic acid, containing at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NanB) in microorganism.

In respect to the above (5), the genetic modification increasing the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism is selected from a) increasing the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism; and/or b) overexpression of UDP-N-Acetyl -D- Glucosamine-2-Epimerase (WecB) in microorganism.

A technician in this field may understand that, to increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism, it may be realized by screening for a gene mutant of UDP-N-Acetyl- D-Glucosamine-2-Epimerase (WecB), encoding UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) with increased activities. The screening for a WecB gene mutant may be achieved by error-prone PCR technology to produce a high-frequency mutant gene. To increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism, it may also be realized by increasing its gene copies or replacing it with a promoter with a higher expression level than that of the natural promoter, for overexpression of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB). In a specific implementation scheme, the microorganism is transformed by molecules of at least recombinant nucleic acid molecules, containing at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism.

In a preferably optimized implementation scheme, the microorganism is transformed by molecules of recombinant nucleic acid, containing one nucleotide sequence encoding UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB).

On the other hand, the nucleotide sequence encoding UDP-N-Acetyl- D-Glucosamine-2-Epimerase (WecB) contains at least one genetic modification that may increase the activities of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB). Preferably the said genetic modification contains one or more substitutions at the following corresponding sites of SEQ ID NO: 50: Substitution of cysteine at Site 34 by serine, substitution of histidine at site 145 by aspartate, substitution of cysteine at Site 226 by phenylalanine, and substitution of valine at Site 245 by glycine. More preferably, the nucleic acid sequence encoding UDP-N-Acetyl-D-Glucosamine-

2-Epimerase (WecB) is SEQ ID NO: 58; the amino acid sequence of the said UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) is SEQ ID NO: 59.

On the other hand, at least approximately 30% (more preferably at least approximately 50%, further more preferably at least approximately 70%, further more preferably at least approximately 80%, and further more at least approximately 90%, and most preferably at least approximately 95%) of the amino acid sequence of the said UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) is the same as that of SEQ ID NO: 50, where the said UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) is active.

On the other hand, the said UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) has the amino acid sequence of SEQ ID NO: 50.

On the other hand, the gene copies in the recombinant nucleic acid molecules, encoding UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB), are increased.

On the other hand, the recombinant nucleic acid molecules contain an endogenous promoter, with a higher expression level of promoters, enhancers, and fusion sequences, etc., than those of natural endogenous promoters. Preferably, the recombinant nucleic acid molecules contains a higher expression level of promoters than that of natural endogenous promoters, such as HCE promoter, gap promoter, trc promoter, and T7 promoter, etc.; more preferably, the recombinant nucleic acid molecules contain trc promoter.

In this Invention, microorganism transformed by recombinant nucleic acid molecules transform are selected from free type (that is to say, recombinant nucleic acid molecules are mounted into plasmids) and integrated type (that is to say, recombinant nucleic acid molecules are integrated into the genomes of microorganism). Preferably, recombinant nucleic acid molecules are integrated into the genomes of microorganism.

In another preferably optimized implementation scheme, the microorganism contains at least one genetic modification of the natural endogenous promoter to the gene encoding UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB). Preferably, the natural endogenous promoter with the gene encoding UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) is replaced by a promoter with a higher expression level, such as HCE promoter, gap promoter, trc promoter, or T7 promoter, etc.; more preferably, the natural endogenous promoter with the gene

encoding UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) is replaced by *trc* promoter.

Based on the preferably optimized implementation scheme, the said microorganism contains one or more of the following genetic modifications:

- 5 (1) Contain at least genetic modification that may decrease the effects of Mannose transporter EIIM, P/III^{man}(ManXYZ) in microorganism;
- (2) Contain at least one genetic modification that may decrease the effects of N-Acetylneuraminate Lyase (NanA) in microorganism;
- (3) Contain at least one genetic modification that may decrease the effects of
10 N-Acetyl-D-Glucosamine-6-Phosphate Deacetylase (NagA) in microorganism;
- (4) Contain at least one genetic modification that may decrease the effects of N-Acetyl-D-Glucosamine Specific Enzyme II^{Nag} (NagE) in microorganism;
- (5) Contain at least one genetic modification that may increase the effects of PhosphoGlucosamine Mutase (GlmM) in microorganism;
- 15 (6) Contain at least one genetic modification that may increase the effects of bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU) in microorganism;

In respect to the above (1), the genetic modifications decreasing the effects of Mannose transporter EIIM, P/III^{man} (ManXYZ) in microorganism include but not
20 limited to: Partial or complete deletion, or partial or complete deactivation of the endogenous gene encoding Mannose transporter EIIM, P/III^{man} (ManXYZ), and/or partial or complete deletion, or partial or complete deactivation of the natural endogenous promoter with the gene encoding Mannose transporter EIIM, P/III^{man} (ManXYZ). Preferably, the genetic modification decreasing the effects of Mannose
25 transporter EIIM, P/III^{man}(ManXYZ) in microorganism is complete deletion (loss) of the endogenous gene encoding EIIM, P/III^{man}(ManXYZ) in microorganism. In a specific implementation scheme, the microorganism is transformed by molecules of at least one recombinant nucleic acid, containing at least one genetic modification that may decrease the effects of Mannose transporter EIIM, P/III^{man} (ManXYZ) in
30 microorganism.

In respect to the above (2), the genetic modifications decreasing the effects of N-Acetylneuraminate Lyase (NanA) in microorganism include but not limited to: Partial or complete deletion, or partial or complete deactivation of the endogenous

gene encoding N-Acetylneuraminate Lyase (NanA), and/or partial or complete deletion, or partial or complete deactivation of the natural endogenous promoter with the gene encoding N-Acetylneuraminate Lyase (NanA). Preferably, the genetic modification decreasing the effects of N-Acetylneuraminate Lyase (NanA) in microorganism is complete deletion (loss) of the natural endogenous gene encoding N-Acetylneuraminate Lyase (NanA) in microorganism. In a specific implementation scheme, the microorganism is transformed by molecules of at least recombinant nucleic acid, containing at least one genetic modification that may decrease the effects of N-Acetylneuraminate Lyase (NanA) in microorganism.

In respect to the above (3), the genetic modifications decreasing the effects of N-Acetyl-D-Glucosamine-6-Phosphate Deactylase (NagA) in microorganism include but not limited to: Partial or complete deletion, or partial or complete deactivation of the endogenous gene encoding N-Acetyl-D-Glucosamine-6-Phosphate Deactylase (NagA), and/or partial or complete deletion, or partial or complete deactivation of the natural endogenous promoter with the gene encoding N-Acetyl-D-Glucosamine-6-Phosphate Deactylase (NagA). Preferably, the genetic modification decreasing the effects of N-Acetyl-D-Glucosamine-6-Phosphate Deactylase (NagA) in microorganism is complete deletion (loss) of the natural endogenous promoter with the gene encoding N-Acetyl-D-Glucosamine-6-Phosphate Deactylase (NagA) in microorganism. In a specific implementation scheme, the microorganism is transformed by molecules of at least one recombinant nucleic acid, containing at least one genetic modification that may decrease the effects of N-Acetyl-D-Glucosamine-6-Phosphate Deactylase (NagA) in microorganism.

In respect to the above (4), the genetic modifications decreasing the effects of N-Acetyl-D-Glucosamine Specific Enzyme II^{Nag} (NagE) in microorganism include but not limited to: Partial or complete deletion, or partial or complete deactivation of the endogenous gene encoding N-Acetyl-D-Glucosamine Specific Enzyme II^{Nag} (NagE), and/or partial or complete deletion, or partial or complete deactivation of the natural endogenous promoter with the gene encoding N-Acetyl-D-Glucosamine Specific Enzyme II^{Nag} (NagE). Preferably, the genetic modification decreasing the effects of N-Acetyl-D-Glucosamine Specific Enzyme IINag (NagE) in microorganism is complete deletion (loss) of the natural endogenous promoter with the gene encoding N-Acetyl-D-Glucosamine Specific Enzyme IINag (NagE) in

microorganism. In a specific implementation scheme, the microorganism is transformed by molecules of at least one recombinant nucleic acid, containing at least one genetic modification that may decrease the effects of N-Acetyl-D-Glucosamine Specific Enzyme II^{Nag} (NagE) in microorganism.

5 In respect to the above (5), the genetic modification increasing the effects of PhosphoGlucosamine Mutase (GlmM) in microorganism is selected from a) increasing the effects of PhosphoGlucosamine Mutase (GlmM) in microorganism; and/or b) overexpression of PhosphoGlucosamine Mutase (GlmM) in microorganism.

A technician in this field may understand that, to increase the effects of
10 PhosphoGlucosamine Mutase (GlmM) in microorganism, it may be realized by screening for a gene mutant of PhosphoGlucosamine Mutase (GlmM), encoding PhosphoGlucosamine Mutase (GlmM) with increased activities. The screening for a GlmM gene mutant may be achieved by error-prone PCR technology to produce a high-frequency mutant gene. To increase the effects of PhosphoGlucosamine Mutase
15 (GlmM) in microorganism, it may also be realized by increasing its gene copies or replacing it with a promoter with a higher expression level than that of the natural promoter, for overexpression of PhosphoGlucosamine Mutase (GlmM). In a specific implementation scheme, the microorganism is transformed by molecules of at least one recombinant nucleic acid, containing at least one genetic modification that may
20 increase the effects of PhosphoGlucosamine Mutase (GlmM) in microorganism.

In a preferably optimized implementation scheme, the microorganism is transformed by molecules of at least recombinant nucleic acid, containing one nucleotide sequence encoding PhosphoGlucosamine Mutase (GlmM).

On the other hand, the nucleotide sequence encoding PhosphoGlucosamine
25 Mutase (GlmM) contains at least one genetic modification that may increase the activities of PhosphoGlucosamine Mutase (GlmM).

On the other hand, the gene copies in the recombinant nucleic acid molecules, encoding PhosphoGlucosamine Mutase (GlmM), are increased.

On the other hand, the recombinant nucleic acid molecules contain an
30 endogenous promoter, with a higher expression level of promoters, enhancers, and fusion sequences, etc., than those of natural endogenous promoters. Preferably, the recombinant nucleic acid molecules contains a higher expression level of promoters than that of natural endogenous promoters, such as HCE promoter, gap promoter, trc

promoter, and T7 promoter, etc.; more preferably, the recombinant nucleic acid molecules contain trc promoter.

In this Invention, microorganism transformed by recombinant nucleic acid molecules transform are selected from free type (that is to say, recombinant nucleic acid molecules are mounted into plasmids) and integrated type (that is to say, recombinant nucleic acid molecules are integrated into the genomes of microorganism). Preferably, recombinant nucleic acid molecules are integrated into the genomes of microorganism.

In another preferably optimized implementation scheme, the microorganism contains at least one genetic modification of the natural endogenous promoter to the gene encoding PhosphoGlucosamine Mutase (GlmM). Preferably, the natural endogenous promoter with the gene encoding PhosphoGlucosamine Mutase (GlmM) is replaced by a promoter with a higher expression level, such as HCE promoter, gap promoter, trc promoter, or T7 promoter, etc.; more preferably, the natural endogenous promoter with the gene encoding PhosphoGlucosamine Mutase (GlmM) is replaced by trc promoter.

In respect to the above (6), the genetic modification increasing the effects of bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU) in microorganism is selected from a) increasing the effects of bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU) in microorganism; and/or b) overexpression of bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU) in microorganism.

A technician in this field may understand that, to increase the effects of bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU) in microorganism, it may be realized by screening for a gene mutant of bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU), encoding of bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU) with increased activities. The screening for a GlmU gene mutant may be achieved by error-prone PCR technology to produce a high-frequency mutant gene. To increase the effects of bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU) in microorganism, it may also be realized by increasing its gene copies or replacing it with a promoter with a higher expression level than that of the natural promoter, for overexpression of bifunctional N-acetyl Glucosamine-1-Phosphate

Uridyltransferase (GlmU). In a specific implementation scheme, the microorganism is transformed by molecules of at least recombinant nucleic acid molecules, containing at least one genetic modification that may increase the effects of bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU) in microorganism.

In a preferably optimized implementation scheme, the microorganism is transformed by molecules of at least recombinant nucleic acid, containing one nucleotide sequence encoding bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU).

On the other hand, the nucleotide sequence encoding bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU) contains at least one genetic modification that may increase the activities of bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU).

On the other hand, the gene copies in the recombinant nucleic acid molecules, encoding bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU), are increased.

On the other hand, the recombinant nucleic acid molecules contain an endogenous promoter, with a higher expression level of promoters, enhancers, and fusion sequences, etc., than those of natural endogenous promoters. Preferably, the recombinant nucleic acid molecules contains a higher expression level of promoters than that of natural endogenous promoters, such as HCE promoter, gap promoter, trc promoter, and T7 promoter, etc.; more preferably, the recombinant nucleic acid molecules contain trc promoter.

In this Invention, microorganism transformed by recombinant nucleic acid molecules transform are selected from free type (that is to say, recombinant nucleic acid molecules are mounted into plasmids) and integrated type (that is to say, recombinant nucleic acid molecules are integrated into the genomes of microorganism). Preferably, recombinant nucleic acid molecules are integrated into the genomes of microorganism.

In another preferably optimized implementation scheme, the microorganism contains at least one genetic modification of the natural endogenous promoter to the gene encoding bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU). Preferably, the natural endogenous promoter with the gene encoding

bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU) is replaced by a promoter with a higher expression level, such as HCE promoter, gap promoter, trc promoter, or T7 promoter, etc.; more preferably, the natural endogenous promoter with the gene encoding bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU) is replaced by trc promoter.

This Invention is further involved with the following preferably optimized implementation schemes:

1. Based on one preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), and at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

2. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), and at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

3. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said

microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), and at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism; and

- 5 B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

Preferably, the said microorganism also contains at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

- 10 4. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

- 15 A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, and at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism; and

- 20 B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

- 25 5. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

- 30 A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism; and

- B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

6. Based on another preferably optimized implementation scheme of the

Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, and at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

7. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, and at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

Preferably, the said microorganism also contains at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

8. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the

effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, and at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

9. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

10. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, and at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

Preferably, the said microorganism also contains at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Synthase

(GlmS) in microorganism.

11. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, and at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

12. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

13. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express

vitroscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism; and

5 B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

Preferably, the said microorganism also contains at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

10 14. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said
15 microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and at least one genetic modification that may
20 increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

25 15. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said
microorganism contains at least one genetic modification that may express
30 vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, and at

least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

5 Preferably, the said microorganism also contains at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

16. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of
10 N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the
15 effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, and at least one
20 genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

17. Based on another preferably optimized implementation scheme of the
25 Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express
30 vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, and at

least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

5 18. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

10 A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and at least one genetic modification that may
15 increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

20 Preferably, the said microorganism also contains at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

25 19. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

30 A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, at least one genetic modification that may increase the effects of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and at least one genetic modification that may increase the effects of

UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

20. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

Preferably, the said microorganism also contains at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

21. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, at least one genetic modification that may increase the effects of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in

microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

22. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, at least one genetic modification that may increase the effects of N-Acetyl- D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

Preferably, the said microorganism also contains at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

23. Based on another preferably optimized implementation scheme of the Invention, the Invention is involved with the method for production of N-Acetyl-D-Glucosamine (GlcNAc) and/or D-Glucosamine Salt by microbial fermentation, which includes:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, at least one genetic modification that may increase the effects of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, at least one genetic modification that may

decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism; and

B) Collection of N-Acetyl-D-Glucosamine (GlcNAc) produced in the cultivation step A).

In the above preferably optimized implementation schemes, further include C) Deacetylation of N-Acetyl-D-Glucosamine (GlcNAc) to produce D-Glucosamine Salt.

In the above preferably optimized implementation schemes, the microorganism further contain: At least one genetic modification that may decrease the effects of Mannose transporter EIIM, P/IIIman (ManXYZ) in microorganism; at least one genetic modification that may decrease the effects of N-Acetylneuraminate Lyase (NanA) in microorganism; at least one genetic modification that may decrease the effects of N-Acetyl-D-Glucosamine-6-Phosphate Deactylase (NagA) in microorganism; at least one genetic modification that may decrease the effects of N-Acetyl-D-Glucosamine Specific Enzyme IINag (NagE) in microorganism.

On one hand for any of the above implementation schemes, the above recombinant nucleic acid molecules may be induced, including but not limited to induction yb lactose; for example, it may realize expression induced by lactase, by adding lactose to the culture solution.

A technician in this field may understand that, various known common fermentation media in this field may be used in this Invention. On the one hand, the fermentation media contain carbon source. On the other hand, the fermentation media contain nitrogen sources. On the other hand, the fermentation media contain both carbon sources and nitrogen sources. On the other hand, the fermentation media contain carbon sources, nitrogen sources, and inorganic salts.

A technician in this field may understand that, various known carbon sources in this field may be used in this Invention, including organic and/or inorganic carbon sources. Preferably, carbon sources are selected from one or more of glucose, fructose, sucrose, galactose, dextrin, glycerin, starch, syrup, and molasses. Preferably, the concentration of a carbon source is maintained in the range of approximately 0.1% - approximately 5%. A technician in this field may understand that, various known nitrogen sources in this field may be used in this Invention, including organic and/or

inorganic nitrogen sources. Preferably, nitrogen sources are selected from one or more of ammonia water, ammonium chloride, ammonium sulfate, ammonium nitrate, ammonium acetate, sodium nitrate, urea, yeast extract, meat extract, peptone, fish meal, bean flour, malt, corn syrup, and cotton seed meal.

5 Preferably, this Invention uses feed-batch fermentation method. Base on one aspect of this Invention, the carbohydrate supplement solution contains glucose and ribose; preferably, the concentration of glucose is 10%-85% (w/v), and that of ribose is 0.5%-15% (w/v); further preferably, the concentration of glucose is 55%-75% (w/v), and that of ribose is 5%-7% (w/v). Base on one aspect of this Invention, the carbohydrate supplement solution contains glucose and gluconate; preferably, the concentration of glucose is 10%-85% (w/v), and that of gluconate is 0.5%-15% (w/v); further preferably, the concentration of glucose is 55%-75% (w/v), and that of gluconate is 2%-3% (w/v); Base on one aspect of this Invention, the carbohydrate supplement solution contains glucose, ribose, and gluconate; preferably, the concentration of glucose is 10%-85% (w/v), that of gluconate is 0.5%-15% (w/v), and that of gluconate is 0.5%-15% (w/v); further preferably, the concentration of glucose is 55%-75% (w/v), that of gluconate is 5%-7% (w/v), and that of gluconate is 2%-3% (w/v); Preferably, gluconate is sodium gluconate.

20 In preferably optimized implementation schemes, the said cultivation step is carried out at approximately 20°C ~ approximately 45°C; further preferably, the said cultivation step is carried out at approximately 33°C ~ approximately 37°C.

 In preferably optimized implementation schemes, the said cultivation stem is carried out at approximately pH4.5 ~ approximately pH8.5. Further preferably, the said cultivation step is carried out at approximately pH6.7 ~ approximately pH7.2.

25 A technician in this field may understand that, various known common methods may be used to collect N-Acetyl-D-Glucosamine in this Invention. Preferably, N-Acetyl-D-Glucosamine may be collected from extracellular products in the fermentation medium. Further preferably, the collection step includes one selected from the following steps: (a) N-Acetyl-D-Glucosamine is precipitated in the fermentation liquid in which microorganism are removed; (b) N-Acetyl-D-Glucosamine is crystallized from the fermentation liquid in which microorganism are removed.

 Based on this Invention, the collection step further includes a decoloration step

of the fermentation liquid. The decoloration step may include but not limited to conduction prior to precipigation or crystallization of the fermentation liquid, and after one or multiple redissolution of precipitates or crystals in the fermentation liquid; the decoloration include activated charcoal treatment and/or chromatographic decoloration. The said chromatographic decoloration includes a step for exposure of the said fermentation liquid to an ion exchange resin. The said ion exchange resins include but not limited to anion exchange resin and/or cation exchange resin; for example, the fermentation liquid is exposed to a mixed bed containing anion and cation exchange resins.

Based on this Invention, N-Acetyl-D-Glucosamine may be deacetylated to produce a D-Glucosamine salt. The said salts include but not limited to hydrochlride, sulfate, sodium salt, phosphate, and bisulfate, etc. For example, N-Acetyl-D-Glucosamine may be deacetylated and hydrolized under acidic and heating conditions a D-Glucosamine salt. Preferably, N-Acetyl-D-Glucosamine may be deacetylated and hydrolized in 30%-70% hydrochloric acid and at 60-90°C to produce N-Glucosamine Hydrochloride; N-Acetyl-D-Glucosamine may also be hydrolyzed under UDP-3-O-N-Acetylglucosamine Deacetylase to produce D-Glucosamine and further to produce a salt.

Based on another implementation scheme of this Invention, this Invention is involved with one microorganism, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb). The above text has described detailedly the genetic modification.

Based on the preferably optimized implementation scheme, the said microorganism contains one or more of the following genetic modifications:

(1) Contain at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism;

(2) Contain at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism;

(3) Contain at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and preferably contain at least one genetic modification that may decrease the effects of Glucosamine-6-Phosphate Synthase (GlmS);

(4) Contain at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, and preferably contain at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NagB)

5 (5) Contain at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism; The above text has described detailedly the genetic modifications.

Based on the preferably optimized implementation scheme, the said microorganism contains one or more of the following genetic modifications:

10 (1) Contain at least genetic modification that may decrease the effects of Mannose transporter EIIM, P/III^{man}(ManXYZ) in microorganism;

(2) Contain at least one genetic modification that may decrease the effects of N-Acetylneuraminate Lyase (NanA) in microorganism;

(3) Contain at least one genetic modification that may decrease the effects of 15 N-Acetyl-D-Glucosamine-6-Phosphate Deactylase (NagA) in microorganism;

(4) Contain at least one genetic modification that may decrease the effects of N-Acetyl-D-Glucosamine Specific Enzyme II^{Nag} (NagE) in microorganism;

(5) Contain at least one genetic modification that may increase the effects of PhosphoGlucosamine Mutase (GlmM) in microorganism;

20 (6) Contain at least one genetic modification that may increase the effects of bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU) in microorganism; The above text has described detailedly the genetic modifications.

This Invention is further involved with the following preferably optimized implementation schemes:

25 1. Based on one preferably optimized implementation scheme of this Invention, this Invention is involved with one microorganism, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), and at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism.

30 2. Based on another preferably optimized implementation scheme of this Invention, this Invention is involved with one microorganism, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), and at least one genetic modification that may

increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism.

3. Based on another preferably optimized implementation scheme of this Invention, this Invention is involved with one microorganism, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), and at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism.

Preferably, the said microorganism also contains at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

4. Based on another preferably optimized implementation scheme of this Invention, this Invention is involved with one microorganism, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, and at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism.

5. Based on another preferably optimized implementation scheme of this Invention, this Invention is involved with one microorganism, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism.

6. Based on another implementation scheme of this Invention, this Invention is involved with one microorganisms, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, and at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism.

7. Based on another implementation scheme of this Invention, this Invention is involved with one microorganisms, where the said microorganism contains: At least

one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, and at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism.

Preferably, the said microorganism also contains at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

8. Based on another implementation scheme of this Invention, this Invention is involved with one microorganisms, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, at least one genetic modification that may increase the effects of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, and at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism.

9. Based on another implementation scheme of this Invention, this Invention is involved with one microorganisms, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism.

10. Based on another implementation scheme of this Invention, this Invention is involved with one microorganisms, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D- Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, and at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism.

Preferably, the said microorganism also contains at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

11. Based on another implementation scheme of this Invention, this Invention is involved with one microorganisms, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, at least one genetic modification that may increase the effects of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, and at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism.

12. Based on another implementation scheme of this Invention, this Invention is involved with one microorganisms, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D- Glucosamine-2-Epimerase (WecB) in microorganism.

13. Based on another implementation scheme of this Invention, this Invention is involved with one microorganisms, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism.

Preferably, the said microorganism also contains at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

14. Based on another implementation scheme of this Invention, this Invention is involved with one microorganisms, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism.

15. Based on another implementation scheme of this Invention, this Invention is involved with one microorganisms, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, and at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism.

Preferably, the said microorganism also contains at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

16. Based on another implementation scheme of this Invention, this Invention is involved with one microorganisms, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, at least one genetic modification that may increase the effects of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, and at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism.

17. Based on another implementation scheme of this Invention, this Invention is involved with one microorganisms, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism.

18. Based on another implementation scheme of this Invention, this Invention is involved with one microorganisms, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine

Kinase (NanK) in microorganism, at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism.

5 Preferably, the said microorganism also contains at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

19. Based on another implementation scheme of this Invention, this Invention is involved with one microorganisms, where the said microorganism contains: At least
10 one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, at least one genetic modification that may increase the effects of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, at least one genetic modification that may decrease the effects of D-Glucosamine-6-
15 Phosphate Deaminase (NagB) in microorganism, and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D- Glucosamine-2-Epimerase (WecB) in microorganism.

20. Based on another implementation scheme of this Invention, this Invention is involved with one microorganisms, where the said microorganism contains: At least
20 one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D- Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and at least one genetic modification that may increase the effects of
25 UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism.

Preferably, the said microorganism also contains at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

21. Based on another implementation scheme of this Invention, this Invention is
30 involved with one microorganisms, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, at least one genetic modification

that may increase the effects of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism.

22. Based on one implementation scheme of this Invention, this Invention is involved with one microorganisms, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism.

Preferably, the said microorganism also contains at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Synthase (GlmS) in microorganism.

23. Based on another implementation scheme of this Invention, this Invention is involved with one microorganisms, where the said microorganism contains: At least one genetic modification that may express vitreoscilla hemoglobin (Vhb), at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism, at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase (NanE) in microorganism, at least one genetic modification that may increase the effects of Glucosamine-6-Phosphate Synthase (GlmS) in microorganism, at least one genetic modification that may decrease the effects of D-Glucosamine-6-Phosphate Deaminase (NagB) in microorganism, and at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism.

In the above preferably optimized implementation schemes, the microorganism further contain: At least one genetic modification that may decrease the effects of Mannose transporter EIIM, P/IIIMan (ManXYZ) in microorganism; at least one

genetic modification that may decrease the effects of N-Acetylneuraminase Lyase (NanA) in microorganism; at least one genetic modification that may decrease the effects of N-Acetyl-D-Glucosamine-6-Phosphate Deacetylase (NagA) in microorganism; at least one genetic modification that may decrease the effects of N-Acetyl-D-Glucosamine Specific Enzyme IINag (NagE) in microorganism.

Based on another implementation scheme of this Invention, this Invention is involved with one vitreoscilla hemoglobin (Vhb) with higher activities, which has the amino acid sequence as shown in SEQ ID NO:65. This Invention is further involved with nucleic acid molecules encoding the above vitreoscilla hemoglobin (Vhb), where the said nucleic acid molecules have the nucleic acid sequence as shown in SEQ ID NO: 64. This Invention is further involved with a carrier containing the above nucleic acid molecules. This Invention is further involved with a microorganism containing the above carrier. This Invention is further involved with a microorganism with genomes containing above nucleic acid molecules.

In this Invention, microorganism may be any one (such as bacteria, protozoan, algae, fungi, or other microorganisms). In a preferably optimized implementation scheme, the microorganisms include but not limited to bacteria, yeast, or fungi. Preferably, the said microorganism is selected from bacteria or yeasts. Further preferably, the bacteria include but not limited to bacteria selected from *Escherichia*, *Bacillus*, *Lactobacillus*, *Pseudomonas*, or *Streptomyces*; more preferably, the bacteria include but not limited to bacteria selected from *Escherichia coli*, *Bacillus subtilis*, *Bacillus licheniformis*, *Lactobacillus brevis*, *Pseudomonas aeruginosa*, or *Streptomyces lividans*. More preferably, the yeasts include but not limited to yeasts selected from *Saccharomyces*, *Schizosaccharomyces*, *Candida*, *Hansenula*, *Pichia*, *Kluyveromyces*, and *Phaffia*; more preferably, the yeasts include but not limited to yeasts selected from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, *Hansenula polymorpha*, *Pichia pastoris*, *Pichia canadensis*, *Kluyveromyces marxianus*, or *Phaffia rhodozyma*. Preferably, the said microorganism is a fungus; more preferably, the said fungi include but not limited to fungi selected from *Aspergillus*, *Absidia*, *Rhizopus*, *Chrysosporium*, *Neurospora*, or *Trichoderma*; more preferably, the fungi include but not limited to those selected from *Aspergillus niger*, *Aspergillus nidulans*, *Absidia coerulea*, *Rhizopus oryzae*, *Chrysosporium lucknowense*, *Neurospora crassa*, *Neurospora intermedia*, or

Trichoderma reesei. Particularly more preferably, the *Escherichia coli* strains include K-12, B, and W, and most preferably, include K-12. Although *Escherichia coli* are used as preferably optimized microorganism, and are used as example of the various implementation schemes of this Invention, it should be understood that this Invention
5 may use any other microorganisms that may produce N-Acetyl-D-Glucosamine and may increase output of N-Acetyl-D-Glucosamine by a genetic modification. The microorganism used in this Invention may also be called as production organism.

In this Invention, the term N-Acetyl-D-Glucosamine may be called as 2-acetamido-2-deoxy-D-glucose. The terms N-Acetyl-D-Glucosamine, N-Acetyl-D-
10 Glucosamine-6-Phosphate and N-Acetyl-D-Glucosamine-1-Phosphate may be abbreviated to GlcNAc, GlcNAc-6-P, and GlcNAc-1-P, respectively. N-Acetyl-D-Glucosamine may also be abbreviated to NAG. Similar to N-Acetyl-D-Glucosamine and its derivatives, the terms D-Glucosamine, D-Glucosamine-6-Phosphate, and D-Glucosamine-1-Phosphate may also be abbreviated to GlcN, GlcN-6-P, and
15 GlcN-1-P. Similarly, the terms N-Acetyl-D-Mannosamine, N-Acetyl-D-Mannosamine-6-Phosphate, glucose, Glucose-6-Phosphate, and Fructose-6-Phosphate may also be abbreviated to ManNAc, ManNAc-6-P, Glc, Glc-6-P, and Fru-6-P, respectively.

The term "increasing the effects of an enzyme in microorganism" means
20 increased activities the enzyme and/or overexpression of the enzyme, thus to increase output of the product manufactured from the substrate catalyzed by the enzyme in microorganism.

The term "decreasing the effects of an enzyme in microorganism" means decreased activities the enzyme and/or reduced expression of the enzyme, thus to
25 decrease output of the product manufactured from the substrate catalyzed by the enzyme in microorganism.

The term "increased activities of an enzyme" means increased capacity of the enzyme to catalyze a certain chemical reaction. It encompasses increased capacity of the enzyme to catalyze a chemical reaction where the inhibition of the enzyme by the
30 product, and the affinity of the enzyme to the substrate remain unchanged, and/or increased capacity resulted from decreased inhibition of the enzyme by the product, and/or from increased affinity of the enzyme to the substrate. The term "decreased inhibition of the enzyme by the product" means decreased activities of the enzyme to

catalyze a reaction, due to specific inhibition of the enzyme by its end product. The term "increased affinity of the enzyme to the substrate" means increased affinity of the enzyme to the substrate to be catalyzed.

In Figure 1, using *Escherichia coli* as example, it illustrates the major aspect of the genetic modification in the Glucosamine metabolic pathway used to manufacture N-Acetyl-D-Glucosamine in large scale, as disclosed in this Invention. In regard to Figure 1, the bold arrow means metabolic produced and/or increased through genetic modification in this Invention. Figure 1 discloses several synthetic methods used to synthesize N-Acetyl-D-Glucosamine, which include a modification to Vhb, may further include a modification to NanK, NagE, NagB, GlmS, or WecB, or their combination, and may also further include a modification to ManXYZ, NanA, NagA, NagE, GlmM, or GlmU, or their combination. A technician in this Field may understand that, other microorganisms have a similar carbohydrate metabolic pathway, and in such a metabolic pathway, the genes and proteins have similar structure and function. Therefore, the contents discussed in this Invention are applicable for both *Escherichia coli* and other microorganisms, and other microorganisms are obviously included in this Invention.

The enzyme known with the same biological activities in this field may have different names, dependent on the microorganism from which the enzyme is originated. Optional names of many enzymes and the name of a specific gene encoding such enzymes are provided below. The names of those enzymes may be used interchangeably, or may be used for a given sequence or organism if appropriate; however, this Invention is intended to include an enzyme with the specific function, originated from any organism.

For example, the enzyme generally called as "N-Acetyl-D-Mannosamine Kinase" in this article catalyzes phosphorylation of N-Acetyl-D-Mannosamine into N-Acetyl-D-Mannosamine-6-P. N-Acetyl-D-Mannosamine Kinase originated from *Escherichia coli* is generally called as NanK. N-Acetyl-D-Mannosamine Kinase originated from various organisms is well known in this field, and may be used in the genetic modification strategy in this Invention. For example, this article describes that N-Acetyl-D-Mannosamine Kinase originated from *Escherichia coli* has the nucleic acid sequence codes represented by SEQ ID NO: 16, and the amino acid sequence represented by SEQ ID NO: 17.

The enzyme generally called as "N-Acetyl-D-Mannosamine-6-P Epimerase" in this article catalyzes transformation of N-Acetyl-D-Mannosamine-6-P into N-Acetyl-D-Glucosamine-6-P. N-Acetyl-D-Mannosamine-6-P Epimerase originated from *Escherichia coli* is generally called as NanE. N-Acetyl-D-Mannosamine-6-P Epimerase originated from various organisms is well known in this field, and may be used in the genetic modification strategy in this Invention. For example, this article describes that N-Acetyl-D-Mannosamine-6-P Epimerase originated from *Escherichia coli* has the nucleic acid sequence codes represented by SEQ ID NO: 28, and the amino acid sequence represented by SEQ ID NO: 29.

The enzyme generally called as "UDP-N-Acetyl-D-Glucosamine-2-Epimerase" in this article catalyzes transformation of UDP-N-Acetyl-D-Glucosamine into N-Acetyl-D-Mannosamine. UDP-N-Acetyl-D-Glucosamine-2-Epimerase originated from *Escherichia coli* is generally called as WecB. UDP-N-Acetyl-D-Glucosamine-2- Epimerase originated from various organisms is well known in this field, and may be used in the genetic modification strategy in this Invention. For example, this article describes that UDP-N-Acetyl-D-Glucosamine-2-Epimerase originated from *Escherichia coli* has the nucleic acid sequence codes represented by SEQ ID NO: 49, and the amino acid sequence represented by SEQ ID NO: 50.

The enzyme generally called as "D-Glucosamine-6-Phosphate Deaminase" in this article catalyzes a reversible reaction of D-Glucosamine-6-Phosphate and water to produce Glucose-6-Phosphate and ammonium. The enzyme is also called as D-Glucosamine-6-Phosphate Epimerase , GlcN6P Deaminase, Phospho-D-Glucosamine Epimerase, Phospho-D- Glucosamine Epimerase, D-Glucosamine Phosphate Ester Deaminase, and 2-Amino-2-Deoxy-D-Glucose-6- Phosphate Ketol Epimerase (deamination). D-Glucosamine-6-Phosphate Deaminase originated from various organisms is well known in this field, and may be used in the genetic modification strategy in this Invention. The enzyme is generally called as NagB in *Escherichia coli* or other bacteria.

The enzyme generally called as "D-Glucosamine-6-Phosphate Synthase" in this article catalyzes Glucose-6-Phosphate and Glutamine to produce D-Glucosamine- 6-Phosphate and glutamic acid. The enzyme is also called as D-Glucosamine-Fructose -6 - Phosphate Aminotransferase (isomerization), Phosphohexose Aminotransferase, D- Fructose-6-Phosphate Transamidase, D-Glucosamine-6-Phosphate Epimerase (to

produce glutamin), L-Glutamine-Fructose-6-Phosphate Transamidase, and GlcN6P Synthase. D-Glucosamine-6-Phosphate Synthase originated from various organisms is well known in this field, and may be used in the genetic modification strategy in this Invention. D-Glucosamine-6-Phosphate Synthase originated from Escherichia coli or other bacteria is generally called as GlmS.

The enzyme generally called as "N-Acetyl-D-Glucosamine-6-Phosphate Deacetylase" may hydrolyze N-Acetyl-D-Glucosamine-6-Phosphate into D-Glucosamine - 6- Phosphate and acetate ester. N-Acetyl-D- Glucosamine-6-Phosphate Deacetylase originated from various organisms is well known in this field, and may be used in the genetic modification strategy in this Invention. For example, this article describes the enzyme called as NagA, originated from Escherichia coli.

The enzyme generally called as "N-Acetylneuraminate Lyase" catalyze degradation of N-Acetyl-D-Mannosamine into N-Acetylneuraminic Acid. N-Acetylneuraminate Lyase originated from various organisms is well known in this field, and may be used in the genetic modification strategy in this Invention. For example, this article describes that N-Acetylneuraminate Lyase originated from Escherichia coli is called as NagA.

The enzyme generally called as "PhosphoGlucosamine Mutase" in this article catalyzes transformation of D-Glucosamine-6-Phosphate into D-Glucosamine-1-Phosphate. Phospho-D-Glucosamine Mutase originated from various organisms is well known in this field and may be used in the genetic modification strategy of this Invention. The enzyme PhosphoGlucosamine Mutase is generally called as GlmM in Escherichia coli or other bacteria.

The enzyme generally called as "D-Glucosamine-1-PhosphateN-Acetyltransferase" in this article catalyzes Transformation of D-Glucosamine-1-Phosphate and Acetyl Coenzyme A into N-Acetyl-D-Glucosamine-1-Phosphate, and release of CoA. As a bifunctional enzyme, it also has the function of N-Acetyl-D-Glucosamine-1-Phosphate Uridyltransferase, and is also called as UDP-N-Acetyl-D-Glucosamine Pyrophosphorylase, UDP-N-Acetyl-D-Glucosamine Diphosphorylase, and catalyze further transformation of N-Acetyl-D-Glucosamine-1-Phosphate into UDP-N-Acetyl-D-Glucosamine. D-Glucosamine-1- PhosphateN-Acetyltransferase and N-Acetyl-D-Glucosamine-1-Phosphate Uridyltransferase

originated from various organisms are well known in this field, and may be used in the genetic modification strategy in this Invention. The enzyme is generally called as GlmU in *Escherichia coli* or other bacteria.

Through a crafty design, "Trc promoter" may be used for prokaryotic expression, such as *Escherichia coli* expression system. Trc promoter is well known in this field and may be used in the genetic modification strategy of this Invention. For example, this article describes that Trc promoter has a nucleotide sequence represented by SEQ ID NO: 32.

As disclosed by the WO2004/003175 Invention, D-Glucosamine is very unstable in the common pH range for growth of *Escherichia coli*. D-Glucosamine and/or its degradation products produce toxic effects to the strains. Toxic effects may be evenly observed when D-Glucosamine in a low concentration of up to 20 g/L is preincubated in the culture medium (pH7.0), prior to cell inoculation. Toxic effects are partially caused by the degradation products of D-Glucosamine in the culture medium with an initial pH 7.0. GlcN is more stable in low pH conditions, and D-Glucosamine may not be degraded below pH4.7. However, *Escherichia coli* grow slowly under pH 6-7 conditions. Therefore, the scheme is difficult to implement production of D-Glucosamine in the fermentation tank under relatively low pH.

Based on this Invention, by expression of vitreoscilla hemoglobin (Vhb) in microorganism, it may increase the microorganism's utilization ability for dissolved oxygen, accelerate synthesis of proteins and metabolic products, promote microbial growth, increase fermentation potency and level. Moreover, D-Glucosamine-6-P (GlcN-6-P) is catalyzed by GlmM and GlmU to produce UDP-N-Acetyl-D-Glucosamine (UDP-GlcNAc) in cells, and is catalyzed by UDP-N-Acetyl-Glucosamine-2-Epimerase (WecB) to produce N-Acetyl-D-Mannosamine (ManNAc). Through overexpression of NanK and NanE, it is further transformed into N-Acetyl-D-Glucosamine-6-Phosphate (GlcNAc-6-P), and dephosphorylated under action of phosphatase and then excreted out of cells to produce N-Acetyl-D-Glucosamine (GlcNAc). The method in this Invention may avoid production of D-Glucosamine, and thus avoid toxic effects of D-Glucosamine and/or its degradation products to the strains.

Therefore, the beneficial effects of this Invention Based on this Invention lie in: This invention has demonstrated that, by expression of vitreoscilla hemoglobin (Vhb)

in microorganism, it may increase the microorganism's utilization ability for dissolved oxygen, accelerate synthesis of proteins and metabolic products, promote microbial growth, and increase fermentation potency and level, so as to produce wholly natural N-Acetyl-D-Glucosamine by microbial fermentation method. The new production method has no risk for contamination by heavy metals, and no risk for residue of antibiotics or drugs; the production is not influenced by supply of raw materials, and may produce stably for a long term, with high yield and low cost; The produced N-Acetyl-D-Glucosamine and D-Glucosamine products are characterized by non-animal source. The production does not use chitin from shrimp shell, and does use glucose, etc., as carbohydrate source for fermentation. The products are vegetarian products, without any allergens of aquatic products.

The whole contents of the public literatures and references cited or described in this article are attached for reference in this article.

15 Descriptions for Attached Figures

Figure 1, Biosynthesis Strategy and Metabolic Engineering Strategy for N-Acetyl-D-Glucosamine in Escherichia Coli

Actual Implementation Modes

Hereafter this Invention is further described detailedly in combination with the implementation examples. The following examples are just used as ones to clarify and explain this Invention, and cannot be explained as any limitation to the protection range of this Invention. The technology realized, based on the contents of this Invention, are covered in the protection range of this Invention.

Unless otherwise specified, the raw materials and reagents used in the implementation examples are commercially available goods.

The list of various modified microorganisms involved in and/or described by this Invention is provided below.

Strain No.	Genotype Description	Remarks
AT-001	ATCC 27325, F-IN(rrnD-rrnE)1 lambda-, a prototrophic derivative strain of Escherichia coli K-12	Parent strain of engineering bacterial, obtained from American Type Culture Collection (ATCC)

Strain No.	Genotype Description	Remarks
AT-002-01	AT-001, Δ manXYZ::fKanrf	Example 1
AT-002-02	AT-001, Δ manXYZ	Example 1
AT-003-01	AT-002-02, Δ nanA::fKanrf	Example 1
AT-003-02	AT-002-02, Δ nanA	Example 1
AT-004-01	AT-003-02, Δ nagA::fKanrf	Example 1
AT-004-02	AT-003-02, Δ nagA	Example 1
AT-005-01	AT-004-02, Δ nagE::fKanrf	Example 1
AT-005-02	AT-004-02, Δ nagE	Example 1
AT-006-01	AT-004-02, Δ nagE::pTrc-nanK-fKanrf	Example 2a
AT-006-02	AT-004-02, Δ nagE::pTrc-nanK	Example 2a
AT-007-01	AT-004-02, Δ nagE::pTrc-nanKM-fKanrf	Example 2b
AT-007-02	AT-004-02, Δ nagE::pTrc-nanKM	Example 2b
AT-052	AT-007-02, vhb/pTrc99A	Example 2.c
AT-053	AT-007-02, vhbM/pTrc99A	Example 2.c
AT-030-01	AT-004-02, Δ nagE::pTrc-nanE-fKanrf	Example 3.a
AT-030-02	AT-004-02, Δ nagE::pTrc-nanE	Example 3.a
AT-031-01	AT-004-02, Δ nagE::pTrc-nanEM-fKanrf	Example 3.b
AT-031-02	AT-004-02, Δ nagE::pTrc-nanEM	Example 3.b
AT-054	AT-031-02, vhb/pTrc99A	Example 3.c
AT-055	AT-031-02, vhbM/pTrc99A	Example 3.c
AT-042-01	AT-004-02, Δ nagE::pTrc-wecB-fKanrf	Example 4.a
AT-042-02	AT-004-02, Δ nagE::pTrc-wecB	Example 4.a
AT-043-01	AT-004-02, Δ nagE::pTrc-wecBM-fKanrf	Example 4.b
AT-043-02	AT-004-02, Δ nagE::pTrc-wecB	Example 4.b
AT-056	AT-043-02, vhb/pTrc99A	Example 4.c
AT-057	AT-043-02, vhbM/pTrc99A	Example 4.c
AT-048	AT-005-02, Δ nagB promotor::Trc promoter	Example 5.a
AT-049	AT-048, Δ glmS promotor	Example 5.a
AT-050	AT-005-02, Δ glmS promotor::Trc promoter	Example 5.b
AT-051	AT-050, Δ nagB promotor	Example 5.b
AT-058	AT-049, vhb/pTrc99A	Example 5.c
AT-059	AT-049, vhbM/pTrc99A	Example 5.c
AT-060	AT-051, vhb/pTrc99A	Example 5.c
AT-061	AT-051, vhbM/pTrc99A	Example 5.c

Strain No.	Genotype Description	Remarks
AT-009	AT-007-02, Δ nanE promotor:: <trc promoter<="" td=""><td>Example 6</td></trc>	Example 6
AT-062	AT-009, vhb/pTrc99A	Example 6
AT-063	AT-009, vhbM/pTrc99A	Example 6
AT-010	AT-007-02, Δ nagB promotor:: <trc promoter<="" td=""><td>Example 7</td></trc>	Example 7
AT-011	AT-010, Δ glmS promotor	Example 7
AT-012	AT-007-02, Δ glmS promotor:: <trc promoter<="" td=""><td>Example 7</td></trc>	Example 7
AT-013	AT-012, Δ nagB promotor	Example 7
AT-064	AT-011, vhb/pTrc99A	Example 7
AT-065	AT-011, vhbM/pTrc99A	Example 7
AT-066	AT-013, vhb/pTrc99A	Example 7
AT-067	AT-013, vhbM/pTrc99A	Example 7
AT-019	AT-007-02, Δ wecB promotor:: <trc promoter<="" td=""><td>Example 8</td></trc>	Example 8
AT-068	AT-019, vhb/pTrc99A	Example 8
AT-069	AT-019, vhbM/pTrc99A	Example 8
AT-032	AT-031-02, Δ nagB promotor:: <trc promoter<="" td=""><td>Example 9</td></trc>	Example 9
AT-033	AT-032, Δ glmS promotor	Example 9
AT-034	AT-031-02, Δ glmS promotor:: <trc promoter<="" td=""><td>Example 9</td></trc>	Example 9
AT-035	AT-034, Δ nagB promotor	Example 9
AT-070	AT-033, vhb/pTrc99A	Example 9
AT-071	AT-033, vhbM/pTrc99A	Example 9
AT-072	AT-035, vhb/pTrc99A	Example 9
AT-073	AT-035, vhbM/pTrc99A	Example 9
AT-037	AT-031-02, Δ wecB promotor:: <trc promoter<="" td=""><td>Example 10</td></trc>	Example 10
AT-074	AT-037, vhb/pTrc99A	Example 10
AT-075	AT-037, vhbM/pTrc99A	Example 10
AT-044	AT-043-02, Δ nagB promotor:: <trc promoter<="" td=""><td>Example 11</td></trc>	Example 11
AT-045	AT-044, Δ glmS promotor	Example 11
AT-046	AT-043-02, Δ glmS promotor:: <trc promoter<="" td=""><td>Example 11</td></trc>	Example 11
AT-047	AT-046, Δ nagB promotor	Example 11
AT-076	AT-045-02, vhb/pTrc99A	Example 11
AT-077	AT-045-02, vhbM/pTrc99A	Example 11
AT-078	AT-047-02, vhb/pTrc99A	Example 11
AT-079	AT-047-02, vhbM/pTrc99A	Example 11
AT-015	AT-011, Δ nanE promotor:: <trc promoter<="" td=""><td>Example 12</td></trc>	Example 12
AT-017	AT-013, Δ nanE promotor:: <trc promoter<="" td=""><td>Example 12</td></trc>	Example 12

Strain No.	Genotype Description	Remarks
AT-027	AT-015, Δ_{wecB} promotor:: <i>Trc</i> promoter	Example 12
AT-029	AT-017, Δ_{wecB} promotor:: <i>Trc</i> promoter	Example 12
AT-080	AT-027, <i>vhb</i> /p <i>Trc99A</i>	Example 12
AT-081	AT-027, <i>vhbM</i> /p <i>Trc99A</i>	Example 12
AT-082	AT-035, <i>vhb</i> /p <i>Trc99A</i>	Example 12
AT-083	AT-035, <i>vhbM</i> /p <i>Trc99A</i>	Example 12

Example 1

This implementation describes construction of a mutant strain of *Escherichia coli*, for which the relevant metabolic pathways for intake of N-Acetyl-D-Glucosamine is blocked and degradation of beneficial intermediate products are blocked.

The said parent strain of the production strain is AT-001 (*Escherichia coli* ATCC 27325), belonging to K-12 derivative strain of *Escherichia coli*, and is obtained from American Type Culture Collection.

Blockage of intake of N-Acetyl-D-Glucosamine into the strain, and of degradation of intermediate products may reduce consumption during metabolism, and increase accumulation of the target product (N-Acetyl-D-Glucosamine).

To construct such mutant host strain, *manXYZ*, *nanA*, *nagA*, and *nagE* gene sequences in its chromosomal genomes may be deleted completely or partially, to deactivate its function, and thus result in accumulation of N-Acetyl-D-Glucosamine.

Such deletion of gene sequence in the chromosome may be accomplished by Red recombinant technology. Red recombination is an homologous recombination technology, based on Red operon in λ phage, and mediated by RecE/RecT system in λ phage. By the technology, it may carry out simply and rapidly multiple modifications such as insertion, knockout, and mutation to any large DNA molecules. Simply speaking, for Red recombination technology, pKD46 plasmids with the recombinase-expressing gene are introduced into thalli, then the prepared linear DNA segment for targeting is electrotransformed, positive clones are screened, and finally the resistance gene in the strain is removed.

The specific operational process is described hereafter:

1. Deletion of *manXYZ* gene sequence

Mannose transporter EIIM, P/III^{man} (mannose transporter EIIM, P/III^{Man} ,

ManXYZ) may be used as a second transporter of N-Acetyl-D-Glucosamine; it may transport hexose such as N-Acetyl-D-Glucosamine into cells, and thus the target product excreted out of cells and accumulated is transported into cells for degradation. Deletion of manXYZ gene sequence may block transportation of extracellular N-Acetyl-D-Glucosamine into cells for degradation.

(1) Preparation of Linear DNA Full-length PCR Segment for Targeting of Red Recombination

1) fKanrf segment amplified by PCR

fKanrf, i.e. FRT-Kanr-FRT segment means a FRT site base sequence for specific recognition of FLP recombinase, mounted at both ends of kalamycin resistance gene (Kanr).

Primer Design: Forward primer (mfKanf-F) SEQ ID No.1, and reverse primer (mfKanf-R) SEQ ID No.2.

Template: pPic9K.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

fKanrf size: 1.28kb. Its nucleotide sequence is SEQ ID No.3.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

2) PCR Amplification of Linear DNA Full-length PCR Segment for Targeting of Red Recombination

Design of Homologous Arm Primer: According to the manXYZ sequence SEQ ID No.4, it is designed to delete the homologous arm forward primer (manXYZKO-F) SEQ ID No.5 and reverse primer (manXYZKO-R) SEQ ID No.6 of the manXYZ sequence.

Template: Amplification of fKanrf PCR Segment.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product: Homologous Arm + fKanrf + Homologous Arm.

The PCR product is separated by agarose gel electrophoresis, and purified and recovered to obtain 100 ng/μL linear DNA full-length PCR segment for targeting of

Red recombination.

(2) Red Recombination Operation

First, pKD46 carrier is introduced into the AT-001 strain of *Escherichia coli*. Then, the prepared linear DNA segment for targeting is electrotransformed, and positive clones are screened. Finally, the resistance gene is removed.

1) Transformation of pKD46 Plasmid

pKD46 carrier is a plasmid with Red recombinase-expressing gene, which is 3-gene segment expressing *Exo*, *Bet*, and *Gam*; the 3 genes are placed below the arabinose promoter, and may express in a large amount by induction of L-arabinose. It is necessary to transform pKD46 plasmid into *Escherichia coli*, to achieve purpose to modify the target gene in the chromosome by Red recombination.

①Preparation of Competence: First, inoculate the bacterial suspension of *Escherichia coli* ATCC 27325 stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h. then add the culture solution to a 10-mL centrifuge tube, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL 0.1M CaCl₂ on an ice bath for 5 min. Finally, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL of 0.1M CaCl₂ on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation. Here, preparation of 0.1M CaCl₂: Prepare a 1M CaCl₂ solution with anhydrous CaCl₂, autoclave at a steam pressure of 15 lbf/in² for 20 min, and dispense 1.5 mL and store at -20°C; for use, allow to thaw, and then dilute in a ratio of 1:10 to produce a 0.1M CaCl₂ solution.

②Plasmid Transformation: Transfer 250 μL of spontaneously sedimented thalli, add 5 μL of pKD46 plasmid, and cultivate at -4°C for 30min. Then heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h. Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate. Cultivate overnight (for 12-16 h) at 30°C. Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification. Store the positive strain for use.

2)Electrotransform linear DNA segment for targeting, and screen positive clone

①Preparation of electrotransformed competence: Inoculate the AT-001 strain of

Escherichia coli ATCC 27325, containing pKD46, into a test tube of LB medium containing Ampicillin (Amp), and shake-cultivate overnight at 250 rpm; **On the next day, inoculate in a ratio of 1% into LB medium containing Amp, and cultivate at 30 °C; when OD₆₀₀ reaches approximately 0.2, add 0.2% L-Arabinose, and**
5 **induce at 30 °C for 35 min until OD₆₀₀ reaches approximately 0.4. Cool on an ice bath. Wash once with ultrapure water, wash twice with 10% glycerin, and finally resuspend with 10% glycerin; the amount of glycerin used is to produce a final concentration of the thalli concentrated by 500-1000 folds.**

②Transformation by electric shock: **Take out a 2-mm electrotransformation**
10 **cup from 70% ethanol, wash with sterilized ultrapure water, and irradiate by UV lamp for 30 min. Precool at 4 °C for 30 min. Transfer 90 μL of finally resuspended cells to a precooled centrifuge tube, add 5 μL (more than 100 ng) of the full-length PCR segment (linear DNA) obtained in Step (1), suction gently by a gun, and maintain on an ice bath for 30 min. Electrotransformation**
15 **Parameters: 2500V, 200Ω, 25μF.**

③Resuscitate and screen positive clones: **add 1 mL of LB broth medium, and cultivate at 37 °C and 100rpm for 1 h. Then smear one kanamycin (Kan) plate with every 200 μL, 5 plates in total. Smear evenly and allow to air-dry. Cultivate at 30 °C for 24 h. Pick up clones grown under kanamycin resistance, and carry**
20 **out PCR identification to screen positive clones.**

No. of the obtained strain: AT-002-01 (AT-001, $\Delta_{\text{manXYZ}}::\text{fKanrf}$).

(3) Removal of the Resistance Gene

To facilitate subsequent work, the resistance gene in the obtained strain (positive clones) may be removed. Removal of the resistance gene may be accomplished by
25 pCP20 plasmid. pCP20 is a plasmid with ampicillin and chloramphenicol resistance genes, and may express FLP recombinase after thermal induction, which may identify specifically FRT sites. The sequence between FRT sites may be deleted by recombination, to retain only one FRT site.

Introduce pCP20 into the above kanamycin-resistant clones, cultivate at 30 °C
30 for 8 h, then increase to 42 °C and cultivate overnight, and thermally induce to express FLP recombinase; the plasmids are lost gradually. Streak the plate of antibiotics-free culture medium by an inoculating loop dipped in the bacterial suspension, pick up grown monoclonal clones and dot on the kanamycin-resistant plate;

those that cannot grow are clones of which the kanamycin resistance gene has been removed by FLP recombination. Carry out PCR with identification primer to identify clones losing kanamycin resistance.

No. of the obtained strain: AT-002-02 (AT-001, Δ_{manXYZ}).

5 2. Deletion of nanA Gene Sequence

N-Acetylneuraminate Lyase (N-acetylneuraminate lyase, NanA) can degrade N-Acetyl-D-Mannosamine (ManNAc) in microorganism to produce N-Acetyl-D-Neuraminic Acid (Neu5Ac). Deletion of the nanA gene sequence in nanKETA operon may block degradation of N-Acetyl-D-Mannosamine (ManNAc) into N-Acetyl-D-Neuraminic Acid (Neu5Ac).

10 (1) Preparation of Linear DNA Full-length PCR Segment for Targeting of Red Recombination

Design of Homologous Arm Primer: According to the nanA sequence SEQ ID No.7 in the former segment of nanE-nanK, the homologous arm primers for deletion of nanA sequence are designed: Forward primer (nanAKO-F) SEQ ID No.8 and reverse primer (nanAKO-R) SEQ ID No.9.

Template: Amplification of fKanrf PCR Segment.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product: Homologous Arm + fKanrf + Homologous Arm.

The PCR product is separated by agarose gel electrophoresis, and purified and recovered to obtain 100 ng/ μ L linear DNA full-length PCR segment for targeting of Red recombination.

25 (2) Red Recombination Operation

First, pKD46 carrier is introduced into the AT-002-02 strain of Escherichia coli. Then, the prepared linear DNA segment for targeting is electrotransformed, and positive clones are screened. Finally, the resistance gene is removed.

1) Transformation of pKD46 Plasmid

□ **Preparation of Competence:** First, inoculate the bacterial suspension of *Escherichia coli* AT-002-02 (AT-001, Δ_{manXYZ}) stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225rpm for 2-3 h. then add the culture solution to a 10-mL centrifuge tube, centrifugate at 4000g $\times$

5min, discard the supernatant, and suspend with 5 mL 0.1M CaCl₂ on an ice bath for 5 min. Finally, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL of 0.1M CaCl₂ on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation.

5 □ **Plasmid Transformation:** Transfer 250 μL of spontaneously sedimented thalli, add 5 μL of pKD46 plasmid, and cultivate at -4°C for 30 min. Then heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h. Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate. Cultivate overnight (for 12-16 h) at 30°C. Pick up monoclonal, add 5 mL of LB broth
10 medium and cultivate, and withdraw plasmid for identification. Store the positive strain for use.

2) Electrotransform linear DNA segment for targeting, and screen positive clone

①Preparation of Electrotransformed Competence: Inoculate the AT-002-02 strain of Escherichia coli, containing pKD46, into a test tube of LB medium containing Ampicillin (Amp), and shake-cultivate at 250 rpm. **On the next day, inoculate in a ratio of 1% into LB medium containing Amp, and cultivate at 30°C; when OD₆₀₀ reaches approximately 0.2, add 0.2% L-Arabinose, and induce at 30°C for 35 min until OD₆₀₀ reaches approximately 0.4. Cool on an ice
15 bath. Wash once with ultrapure water, wash twice with 10% glycerin, and finally resuspend with 10% glycerin; the amount of glycerin used is to produce a final concentration of the thalli concentrated by 500-1000 folds.**

②Transformation by electric shock: **Take out a 2-mm electrotransformation cup from 70% ethanol, wash with sterilized ultrapure water, and irradiate by
25 UV lamp for 30 min. Precool at 4°C for 30 min. Transfer 90 μL of finally resuspended cells to a precooled centrifuge tube, add 5 μL (more than 100 ng) of the full-length PCR segment (linear DNA) obtained in Step (1), suction gently by a gun, and maintain on an ice bath for 30 min. Electrotransformation Parameters: 2500V, 200Ω, 25μF.**

30 ③Resuscitate and screen positive clones: **add 1 mL of LB broth medium, and cultivate at 37°C and 100rpm for 1 h. Then smear one kanamycin (Kan) plate with every 200 μL, 5 plates in total. Smear evenly and allow to air-dry. Cultivate**

at 30°C for 24 h. Pick up clones grown under kanamycin resistance, and carry out PCR identification to screen positive clones.

No. of the obtained strain: AT-003-01 (AT-002-02, $\Delta_{nanA}::fKanrf$).

(3) Removal of the Resistance Gene

5 Introduce pCP20 into the above kanamycin-resistant clones, cultivate at 30°C for 8 h, then increase to 42°C and cultivate overnight, and thermally induce to express FLP recombinase; the plasmids are lost gradually. Streak the plate of antibiotics-free culture medium by an inoculating loop dipped in the bacterial suspension, pick up grown monoclonal and dot on the kanamycin-resistant plate;
10 those that cannot grow are clones of which the kanamycin resistance gene has been removed by FLP recombination. Carry out PCR with identification primer to identify clones losing kanamycin resistance.

No. of the obtained strain: AT-003-02 (AT-002-02, Δ_{nanA}).

3. Deletion of nagA Gene Sequence

15 N-Acetyl-D-Glucosamine-6-Phosphate Deacetylase (NagA) can transform N-Acetyl-D-Glucosamine-6-Phosphate (GlcNAc-6-P) in microorganism into D-Glucosamine-6-Phosphate (GlcN-6-P). Deletion of the nagA gene sequence in nag operon (nagE-nagBACD) may block transformation of
20 N-Acetyl-D-Glucosamine-6-Phosphate (GlcNAc-6-P) into D-Glucosamine-6-Phosphate (GlcN-6-P).

(1) Preparation of Linear DNA Full-length PCR Segment for Targeting of Red Recombination

Design of Homologous Arm Primers: From NCBI, look up NC_000913, nagA sequence SEQ ID No.10 for N-Acetyl-D-Glucosamine-6-Phosphate Deacetylase,
25 *Escherichia coli* str. K-12, design the homologous arm primers for deletion of the nagA gene sequence: Forward primer (nagAKO-F) SEQ ID No.11 and reverse primer (nagAKO-R) SEQ ID No.12.

Template: Amplification of fKanrf PCR Segment.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate
30 at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product: Homologous Arm + fKanrf + Homologous Arm.

The PCR product is separated by agarose gel electrophoresis, and purified and

recovered to obtain 100 ng/ μ L linear DNA full-length PCR segment for targeting of Red recombination.

(2) Red Recombination Operation

First, pKD46 carrier is introduced into the AT-003-02 strain of *Escherichia coli*. Then, the prepared linear DNA segment for targeting is electrotransformed, and positive clones are screened. Finally, the resistance gene is removed.

1) Transformation of pKD46 Plasmid

① Preparation of Competence: First, inoculate the bacterial suspension of *Escherichia coli* AT-003-02 (AT-002-02, Δ nanA) stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h. then add the culture solution to a 10-mL centrifuge tube, centrifugate at $4000g \times 5\text{min}$, discard the supernatant, and suspend with 5 mL 0.1M CaCl_2 on an ice bath for 5 min. Finally, centrifugate at $4000g \times 5\text{min}$, discard the supernatant, and suspend with 5 mL of 0.1M CaCl_2 on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation.

② Plasmid Transformation: Transfer 250 μL of spontaneously sedimented thalli, add 5 μL of pKD46 plasmid, and cultivate at -4°C for 30min. Then heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h. Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate. Cultivate overnight (for 12-16 h) at 30°C . Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification. Store the positive strain for use.

2) Electrotransform linear DNA segment for targeting, and screen positive clone

① Preparation of Electrotransformed Competence: Inoculate the AT-003-02 strain of *Escherichia coli*, containing pKD46, into a test tube of LB medium containing Ampicillin (Amp), and shake-cultivate at 250 rpm, on the next day, inoculate in a ratio of 1% into LB medium containing Amp, and cultivate at 30°C ; when OD600 reaches approximately 0.2, add 0.2% L-Arabinose, and induce at 30°C for 35 min until OD600 reaches approximately 0.4. Cool on an ice bath. Wash once with ultrapure water, wash twice with 10% glycerin, and finally resuspend with 10% glycerin; the amount of glycerin used is to produce a final concentration of the thalli concentrated by 500-1000 folds.

□ Transformation by electric shock: **Take out a 2-mm electrotransformation cup from 70% ethanol, wash with sterilized ultrapure water, and irradiate by UV lamp for 30 min. Precool at 4°C for 30 min. Transfer 90 µL of finally resuspended cells to a precooled centrifuge tube, add 5 µL (more than 100 ng) of the full-length PCR segment (linear DNA) obtained in Step (1), suction gently by a gun, and maintain on an ice bath for 30 min. Electrotransformation Parameters: 2500V, 200Ω, 25µF.**

□ Resuscitate and screen positive clones: **add 1 mL of LB broth medium, and cultivate at 37°C and 100rpm for 1 h. Then smear one kanamycin (Kan) plate with every 200 µL, 5 plates in total. Smear evenly and allow to air-dry. Cultivate at 30°C for 24 h. Pick up clones grown under kanamycin resistance, and carry out PCR identification to screen positive clones.**

No. of the obtained strain: AT-004-01 (AT-003-02, Δ nagA::fKanrf).

(3) Removal of the Resistance Gene

Introduce pCP20 into the above kanamycin-resistant clones, cultivate at 30°C for 8 h, then increase to 42°C and cultivate overnight, and thermally induce to express FLP recombinase; the plasmids are lost gradually. Streak the plate of antibiotics-free culture medium by an inoculating loop dipped in the bacterial suspension, pick up grown monoclonal colonies and dot on the kanamycin-resistant plate; those that cannot grow are clones of which the kanamycin resistance gene has been removed by FLP recombination. Carry out PCR with identification primer to identify clones losing kanamycin resistance.

No. of the obtained strain: AT-004-02 (AT-003-02, Δ nagA).

4. Deletion of nagE Gene Sequence

Deletion of the gene sequence nagE for N-Acetyl-D-Glucosamine Specific Enzyme II^{Nag} (NagE) may block transport of extracellular GlcNAc into cells for degradation.

(1) Preparation of Linear DNA Full-length PCR Segment for Targeting of Red Recombination

Design of Homologous Arm Primers: From NCBI, look up NC_000913, nagB promoter and nagA gene sequence SEQ ID No.13, *Escherichia coli* str. K-12, and design the homologous arm primers for deletion of the nagA gene sequence: Forward primer (nagEKO-F1) SEQ ID No.14 and reverse primer (nagEKO-R1) SEQ ID

No.15.

Template: Amplification of fKanrf PCR Segment.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3:
5 Extend at 72°C for 10 min.

Amplification Product: Homologous Arm + fKanrf + Homologous Arm.

The PCR product is separated by agarose gel electrophoresis, and purified and recovered to obtain 100 ng/μL linear DNA full-length PCR segment for targeting of Red recombination.

10 (2)Red Recombination Operation

First, pKD46 carrier is introduced into the AT-004-02 strain of Escherichia coli. Then, the prepared linear DNA segment for targeting is electrotransformed, and positive clones are screened. Finally, the resistance gene is removed.

1) Transformation of pKD46 Plasmid

15 □ **Preparation of Competence:** First, inoculate the bacterial suspension of *Escherichia coli* AT-004-02 (AT-003-02, ΔnagA) stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h. then add the culture solution to a 10-mL centrifuge tube, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL 0.1M CaCl₂ on an ice bath for
20 5 min. Finally, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL of 0.1M CaCl₂ on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation.

□ **Plasmid Transformation: Transfer 250 μL of spontaneously sedimented thalli, add 5 μL of pKD46 plasmid, and cultivate at -4°C for 30 min. Then heat
25 on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h. Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate. Cultivate overnight (for 12-16 h) at 30°C. Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification. Store the positive strain for use.**

30 2) Electrotransform linear DNA segment for targeting, and screen positive clone

□ **Preparation of Electrotransformed Competence: Inoculate the AT-004-02 strain of Escherichia coli, containing pKD46, into a test tube of LB medium**

containing Ampicillin (Amp), and shake-cultivate at 250 rpm, on the next day, inoculate in a ratio of 1% into LB medium containing Amp, and cultivate at 30°C; when OD600 reaches approximately 0.2, add 0.2% L-Arabinose, and induce at 30°C for 35 min until OD600 reaches approximately 0.4. Cool on an ice bath. Wash once with ultrapure water, wash twice with 10% glycerin, and finally resuspend with 10% glycerin; the amount of glycerin used is to produce a final concentration of the thalli concentrated by 500-1000 folds.

□ Transformation by electric shock: Take out a 2-mm electrotransformation cup from 70% ethanol, wash with sterilized ultrapure water, and irradiate by UV lamp for 30 min. Precool at 4°C for 30 min. Transfer 90 µL of finally resuspended cells to a precooled centrifuge tube, add 5 µL (more than 100 ng) of the full-length PCR segment (linear DNA) obtained in Step (1), suction gently by a gun, and maintain on an ice bath for 30 min. Electrotransformation Parameters: 2500V, 200Ω, 25µF.

□ Resuscitate and screen positive clones: add 1 mL of LB broth medium, and cultivate at 37°C and 100rpm for 1 h. Then smear one kanamycin (Kan) plate with every 200 µL, 5 plates in total. Smear evenly and allow to air-dry. Cultivate at 30°C for 24 h. Pick up clones grown under kanamycin resistance, and carry out PCR identification to screen positive clones.

No. of the obtained strain: AT-005-01 (AT-004-02, $\Delta_{\text{nagE}}::\text{fKanrf}$).

(3) Removal of the Resistance Gene

Introduce pCP20 into the above kanamycin-resistant clones, cultivate at 30°C for 8 h, then increase to 42°C and cultivate overnight, and thermally induce to express FLP recombinase; the plasmids are lost gradually. Streak the plate of antibiotics-free culture medium by an inoculating loop dipped in the bacterial suspension, pick up grown monoclonal clones and dot on the kanamycin-resistant plate; those that cannot grow are clones of which the kanamycin resistance gene has been removed by FLP recombination. Carry out PCR with identification primer to identify clones losing kanamycin resistance.

No. of the obtained strain: AT-005-02 (AT-004-02, Δ_{nagE}).

Example 2.a

This example describes gene nanK cloning of N-Acetyl-D-Mannosamine Kinase

(NanK), and transformed nanK/pTrc99A plasmids in *Escherichia coli*, as well as integration of ptrc- nanK gene cassette into the chromosome of *Escherichia coli*.

1. nanK Gene Cloning, Transformation of nanK/pTrc99A plasmid in *Escherichia coli*, and its Influence on the Output of N-Acetyl-D-Glucosamine

5 Amplification of the gene nanK of *Escherichia coli* NanK (N-acetylmannosamine kinase, N-Acetyl-D-Mannosamine Kinase), that is controlled by Trc promoter to transform the strain for overexpression of the enzyme, may strengthen phosphorylation of ManNAc (N-Acetyl-D-mannosamine, N-Acetyl-D-Mannosamine or N-Acetyl-D-Mannosamine) into ManNAc-6-P
10 (N-Acetyl-D-mannosamine-6-P, N-Acetyl-D-Mannosamine-6-Phosphate).

1) nanK Gene Cloning of *Escherichia coli*

From NCBI, look up U00096, to obtain the nucleotide sequence SEQ ID No.16 of the nanK gene of *Escherichia coli*, which amino acid sequence is SEQ ID No.17.

Primer Design: Forward primer (nanK-F) SEQ ID No.18 and reverse primer
15 (nanK-R) SEQ ID No.19.

Template: *Escherichia coli* AT-001.

PCR reaction conditions: Step 1: Denature at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

20 Amplification Product Size: 0.9kb.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

Ligate the obtained PCR amplification segment with pUC57-T carrier, and carry out sequencing for identification, to obtain nanK/ pUC57.

25 2) Construction and Transformation of Plasmid in which nanK gene is controlled by Trc promoter

①Plasmid Construction: **Amplify plasmid nanK/ pUC57, digest enzymatically nanK/pUC57 and carrier pTrc99A with *Nco I* and *Hind III*, separate by agarose gel electrophoresis, purify to recover nanK segment and
30 pTrc99A segment, ligate overnight with T4 DNA ligase at 16°C, and carry out identification to obtain nanK/pTrc99A plasmid.**

②Preparation of Competence: **First, inoculate the bacterial suspension of**

AT-005-02 stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h. then add the culture solution to a 10-mL centrifuge tube, centrifugate at $4000g \times 5\text{min}$, discard the supernatant, and suspend with 5 mL 0.1M CaCl_2 on an ice bath for 5 min. Finally, centrifugate at $4000g \times 5\text{min}$, discard the supernatant, and suspend with 5 mL of 0.1M CaCl_2 on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation.

③Plasmid Transformation: Transfer 250 μL of spontaneously sedimented thalli, add 5 μL of nanK/pTrc99A plasmid, and cultivate at -4°C for 30min. Then heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h. Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate. Cultivate overnight (for 12-16 h) at 30°C . Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification. Store the positive strain for use. Obtain Recombinant Strain nanK/pTrc99A (AT-005-02)

3) Influence of nanK/pTrc99A plasmid transformation on the output of N-Acetyl-D-Glucosamine

Carry out a shake-flask fermentation trial with the recombinant strain and nanK/pTrc99A (AT-005-02) reference strain. Transfer the monoclonal strain freshly cultivated in culture medium of the LB plate, inoculate into a test tube ($13 \times 150\text{mm}$) containing 3 mL of the LB broth culture medium, and shake-cultivate at 30°C and 225rpm for 8 h. Components of LB Broth Medium: 5 g/L yeast powder, 10 g/L peptone, and 10 g/L NaCl. Then transfer the seed culture solution, inoculate in 3% into a 250-mL shake-flask containing 50 mL of the fermentation culture solution (M9 culture solution). The initial OD_{600} is approximately 0.5; shake-cultivate at 37°C and 225rpm. The fermentation cycle is 72 h. At 24 h and 48 h, adjust to pH 7.0 with 10M NaOH. According to glucose consumption in the fermentation broth, add 65% glucose solution to maintain a glucose concentration of 20 g/L. After completion of fermentation, transfer 1 mL of the fermentation broth and centrifugate. Measure the content of N-Acetyl-D-Glucosamine by HPLC method.

①HPLC Method to Measure the Content of N-Acetyl-D-Glucosamine

Buffer: Add 3.5 g of dipotassium hydrogen phosphate to 1-L volumetric flask,

add water to dissolve, add 0.25 mL of ammonia water, then dilute with water and mix well, adjust to pH 7.5, and add water to volume.

Mobile Phase: Acetonitrile:Buffer (75:25).

Diluent: Acetonitrile and water (50:50).

5 Standard Solution: Dissolve 1.0 mg/mL USP N-Acetyl-D-Glucosamine Reference Substance (RS) in the diluent.

Sample Solution: Dissolve 1.0 mg/mL N-Acetyl-D-Glucosamine sample in the diluent.

Liquid Phase Conditions:

10 Model: LC

Detector: UV 195 nm

Chromatographic Column: 4.6-mm×15-cm; 3-μm packing L8

Flow Rate: 1.5 mL/min

Column Temperature: 35°C

15 Injection Volume: 10 μL

②Preparation of M9 Culture Solution

First prepare 5×M9 culture medium: To approximately 800 mL of double distilled water (ddH₂O), add 64 g of Na₂HPO₄·7H₂O, 15g of KH₂PO₄, 2.5g of NaCl, and 5.0 g of NH₄Cl, and after dissolution, add water to 1000 mL. Sterilize at 121°C
20 for 30 min. Then prepare 1M MgSO₄, 1M CaCl₂, and 20% glucose, respectively, and sterilize them separately. Then prepare M9 culture solution according to Table 1, while 1000× microelement solution is prepared according to Table 2.

Table 1. Components of M9 Culture Solution

Ingredients	Amount used (mL/L)
5×M9	200
1M MgSO ₄	2
1M CaCl ₂	0.1
20% Glucose	20
1000× Microelement Solution	1
ddH ₂ O	to 1000

pH	6.9
----	-----

Table 2, Components of 1000× Microelement Solution

Ingredients	Amount used (g/L)
CoCl ₂ ·6H ₂ O	0.01
CuSO ₄ ·5H ₂ O	0.01
MnSO ₄ ·H ₂ O	0.033
FeSO ₄ ·7H ₂ O	0.50
ZnSO ₄ ·7H ₂ O	0.38
H ₃ BO ₃	0.01
NaMoO ₄ ·2H ₂ O	0.01
pH	3

③Influence of nanK/pTrc99A plasmid transformation on the output of N-Acetyl-D-Glucosamine from shake-flask fermentation

See Table 3 for the output from shake-flask fermentation. The results show that: The output by the reference strain AT-005-02 is very low and is not detected, while the output from overexpression by nanK gene of nanK/pTrc99A (AT-005-02), controlled by Trc promoter, is increased obviously.

Table 3. Output from Shake-flask Fermentation by the Recombinant nanK/pTrc99A (AT-005-02)

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-005-02 (AT-004-02, Δ nagE) (Reference)	Not detected
NanK/pTrc99A (AT-005-02)	2.9±0.4

2.Integration of pTrc-nanK Gene Cassette into the Chromosome of Escherichia Coli

Using the nagE gene site as integration site of pTrc-nanK gene cassette into the chromosome. To achieve integration of pTrc-nanK gene cassette into the chromosome of Escherichia coli, first amplify the nanK segment (i.e. pTrc-nanK) with Trc promoter, as well as the kanamycin resistance gene segment with FLP recognition site (FRT site) at its both ends: FRT-Kan^r-FRT (fKan^rf), and splice them.

Then design homologous primers for deletion of the nagE gene sequence, and using the spliced segment of pTrc-nanK and fKanrf as template, amplify the linear DNA full-length segment for targeting of Red recombination.

The specific operating process is provided below:

5 (1) PCR Amplification of pTrc-nanK segment

Template: nanK/pTrc99A.

Primer Design: Forward primer (Trcff-F) SEQ ID No.20, and reverse primer (Trcff-R) SEQ ID No.21.

10 PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Produce Size: 1.05kb.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

15 (2)fKanrf segment amplified by PCR

Primer Design: Forward primer (mfKanf-F) SEQ ID No.1, and reverse primer (mfKanf-R) SEQ ID No.2.

Template: pPic9K.

20 PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

fKanrf size: 1.28kb. Its nucleotide sequence is SEQ ID No.3.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

25 (3) Amplification of fKanrf spliced with pTrc-nanK

Primer Design: Forward primer (fKanf-F) SEQ ID No.22, and reverse primer (fKanf-R) SEQ ID No.23.

Template: fKanrf.

30 PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

fKanrf size from secondary amplificatino: 1.3kb.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

(4)Preparation of Linear DNA Full-length PCR Segment for Targeting of Red Recombination

5 Design of Homologous Arm Primers: Design again homologous arm primers for deletion of the nagE gene sequence: Forward primer (nagEKO-F2) SEQ ID No.24, and reverse primer (nagEKO-R2) SEQ ID No.25.

Template: Mix pTrc-nanK PCR segment, and fKanrf PCR segment from secondary amplification in the ratio of 1:1.

10 PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product: Homologous Arm + pTrc-nanK-fKanrf + Homologous Arm

15 The PCR product is separated by agarose gel electrophoresis, and purified and recovered to obtain 100 ng/μL linear DNA full-length PCR segment for targeting of Red recombination.

(5)Red Recombination Operation

20 **First, pKD46 carrier is introduced into the AT-004-02 strain of Escherichia coli. Then, the prepared linear DNA segment for targeting is electrotransformed, and positive clones are screened. Finally, the resistance gene is removed.**

1) Transformation of pKD46 Plasmid

25 ①Preparation of Competence: **First, inoculate the bacterial suspension of *Escherichia coli* AT-004-02 stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h. then add the culture solution to a 10-mL centrifuge tube, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL 0.1M CaCl₂ on an ice bath for 5 min. Finally, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL of 0.1M CaCl₂ on an ice bath. Allow to stand at -4°C for 12 h for**
30 **spontaneous sedimentation.**

②Plasmid Transformation: **Transfer 250 μL of spontaneously sedimented thalli, add 5 μL of pKD46 plasmid, and cultivate at -4°C for 30min. Then heat**

on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h. Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate. Cultivate overnight (for 12-16 h) at 30°C. Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification. Store the positive strain for use.

2) Electrotransform linear DNA segment for targeting, and screen positive clone

①Preparation of Electrotransformed Competence: Inoculate the AT-004-02 strain of Escherichia coli, containing pKD46, into a test tube of LB medium containing Ampicillin (Amp), and shake-cultivate at 250 rpm, on the next day, inoculate in a ratio of 1% into LB medium containing Amp, and cultivate at 30°C; when OD600 reaches approximately 0.2, add 0.2% L-Arabinose, and induce at 30°C for 35 min until OD600 reaches approximately 0.4. Cool on an ice bath. Wash once with ultrapure water, wash twice with 10% glycerin, and finally resuspend with 10% glycerin; the amount of glycerin used is to produce a final concentration of the thalli concentrated by 500-1000 folds.

②Transformation by electric shock: Take out a 2-mm electrotransformation cup from 70% ethanol, wash with sterilized ultrapure water, and irradiate by UV lamp for 30 min. Precool at 4°C for 30 min. Transfer 90 µL of finally resuspended cells to a precooled centrifuge tube, add 5 µL (more than 100 ng) of the full-length PCR segment (linear DNA) obtained in Step (4), suction gently by a gun, and maintain on an ice bath for 30 min. Electrotransformation Parameters: 2500V, 200Ω, 25µF.

③Resuscitate and screen positive clones: add 1 mL of LB broth medium, and cultivate at 37°C and 100rpm for 1 h. Then smear one kanamycin (Kan) plate with every 200 µL, 5 plates in total. Smear evenly and allow to air-dry. Cultivate at 30°C for 24 h. Pick up clones grown under kanamycin resistance, and carry out PCR identification to screen positive clones.

No. of the obtained strain: AT-006-01 (AT-004-02, $\Delta_{nagE}::pTrc-nanK-fKanrf$).

(6) Removal of the Resistance Gene

Introduce pCP20 into the above kanamycin-resistant clones, cultivate at 30°C for 8 h, then increase to 42°C and cultivate overnight, and thermally induce to

express FLP recombinase; the plasmids are lost gradually. Streak the plate of antibiotics-free culture medium by an inoculating loop dipped in the bacterial suspension, pick up grown monoclones and dot on the kanamycin-resistant plate; those that cannot grown are clones of which the kanamycin resistance gene has been removed by FLP recombination. Carry out PCR with identification primer to identify clones losing kanamycin resistance.

No. of the obtained strain: AT-006-02 (AT-004-02, Δ nagE::pTrc-nanK).

3. Influence of pTrc-nanK gene cassette integration on the output of N-Acetyl-D-Glucosamine

Carry out a shake-flask fermentation trial with the recombinant strain AT-006-02 of which the nagE gene site in the chromosome is integrated with pTrc-nanK gene cassette, and the reference strain. Transfer the monoclonal strain freshly cultivated in culture medium of the LB plate, inoculate into a test tube (13×150mm) containing 3 mL of the LB broth culture medium, and shake-cultivate at 30°C and 225rpm for 8 h. Then transfer the seed culture solution, inoculate in 3% into a 250-mL shake-flask containing 50 mL of the fermentation culture solution (M9 culture solution). The initial OD600 is approximately 0.5; shake-cultivate at 37°C and 225rpm. The fermentation cycle is 72 h. At 24 h and 48 h, adjust to pH 7.0 with 10M NaOH. According to glucose consumption in the fermentation broth, add 65% glucose solution to maintain a glucose concentration of 20 g/L. After completion of fermentation, transfer 1 mL of the fermentation broth and centrifugate. Measure the content of N-Acetyl-D-Glucosamine by HPLC method.

See Table 4 for the output from shake-flask fermentation. The results show that: The outputs by the reference strains AT-001 and AT-005-02 are very low and are not detected, while the output by the recombinant strain integrated with pTrc-nanK gene cassette is increased obviously, and is also increased significantly than that by the recombinant strain nanK/pTrc99A (AT-005-02) not integrated with the gene cassette.

Table 4, Output from Shake-flask Fermentation by the Recombinant Strain integrated with pTrc-nanK Gene Cassette

Species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-001 (Reference)	Not detected
AT-005-02 (AT-004-02, Δ nagE) (Reference)	Not detected

nanK/pTrc99A (AT-005-02)	2.8±0.5
AT-006-02 (AT-004-02, Δ nagE::pTrc-nanK)	4.2±0.5

Example 2.b

This implementation example describes screening for a gene mutant of N-Acetyl-D-Mannosamine Kinase (NanK); the said gene encodes N-Acetyl-D-Mannosamine Kinase (NanK) with increased activities.

To further increase synthetic quantity of N-Acetyl-D-Glucosamine by the production strain, screen a gene mutant encoding N-Acetyl-D-Mannosamine Kinase (NanK) with increased activities. To achieve the purpose, amplify the cloned gene by error-prone PCR technology; used for amplified DNA polymerase, amplify the said gene under conditions leading to high-frequency mismatch, so as to obtain a high-frequency mutation in PCR products.

The specific operating process is provided below:

1. Amplification of the Gene nanK of N-Acetyl-D-Mannosamine Kinase in *Escherichia coli* by Error-prone PCR

By means of Taq DNA polymerase without the property of 3' -5' proofreading function, control the frequency of random mutation under high magnesium ion concentration (8 mmol/L) and different dNTP concentrations (where, the concentration of dATP and dGTP is 1.5 mmol/L; and the concentration of dTTP and dCTP is 3.0 mmol/L), introduce random mutations into the target gene, and construct a mutant library; the template concentration A260 value is 1000 ng/mL, the enzyme concentration is 5 U/ μ L, and the primer concentration is 100 μ M.

Error-prone PCR reaction system (50 μ L): 10 $\times$ PCR reaction buffer 5 μ L, dNTP (2.5 mM) 5 μ L, MgCl₂ (25 mM) 5 μ L, Forward primer (nanK-F, SEQ ID No.18) 1 μ L, reverse primer (nanK-R, SEQ ID No.19) 1 μ L, DNA template (nanK/pUC57) 0.1 μ L, Taq DNA polymerase 0.5 μ L, and ddH₂O 32.4 μ L.

PCR procedure: Predegenerate at 96°C for 4 min; degenerate at 94°C for 1 min, anneal at 56°C for 1 min, extend at 75°C for 2 min, and repeat for 45 cycles; finally extend at 75°C for 15 min, recover PCR product (product size: 0.9 kb) by gel recovery method; transfer 5 μ L of the product and carry out 1% agarose gel electrophoresis test; the product is store at -20°C for use.

2. Construction of the gene mutant library of N-Acetyl-D-Mannosamine Kinase

Digest the above PCR product by two enzymes of restriction endonuclease *Nco I* and *Hind III*, carry out a ligation reaction with pTrc99A digested by *Nco I* and *Hind III*, then transform *Escherichia coli* AT-005-02 with the mixture of the ligated products to obtain a large amount of cloned transformants, and construct a mutant library of transformed thalli.

3. Screening for Mutants with High Enzyme Activities

Using the wild type NanK/pTrc99A (AT-005-02) as reference, pick up randomly 300 mutant clones from the mutant library of transformed thalli, inoculate into 5 mL of LB medium containing 50 µg/mL Ampicillin (Amp), shake-cultivate at 37°C and 150 rpm for 18 h, and then centrifugate at 10000 rpm for 5 min and collect thalli. Discard the supernatant, then resuspend at 4°C in 1 mL of PBS solution (pH 7.5, 10 mmol/L), carry out ultrasonicate at a voltage of 300 V for 10 min (ultrasonicate for 3 s and pause for 6 s), centrifugate, transfer the supernatant as crude extract of enzyme, and carry out a method for determination of enzyme activity.

Determination of N-Acetyl-D-Mannosamine Kinase (NanK) Activity: Based on the phosphorylated amount of N-Acetyl-D-Mannosamine (ManNAc); that is to say, using the reduced amount of N-Acetyl-D-Mannosamine as test marker. Definition of Enzyme Activity Unit: Under the enzymatic reaction conditions, the enzyme amount reduced by the reducing sugar, equivalent to 1 µmol N-Acetyl-D-Mannosamine per minute, is defined as one enzyme activity unit (IU). The specific procedure is provided as follows: Transfer 5 mL of the reaction system as the system for determination of enzyme activity, which contains 500 mmol/L N-Acetyl-D-Mannosamine, 5 mmol/L glucose, 100 mmol/L Tris-HCl (pH8.0), and 100 µL of crude enzyme solution. Carry out the reaction for determination of enzyme activity on a water bath at 37°C, maintain for 4 h, and then place the enzymatic hydrolysate at 70°C for 10 min to stop the reaction. Centrifugate at 3000 rpm for 10 min, and transfer the supernatant. Measure the content of N-Acetyl-D-Mannosamine by HPLC method.

The results show that: The enzyme activity of the mutant strain with maximum activity is 77.5 IU/mL, and the enzyme activity of the reference control is 16.3 IU/mL. Transform NanK by error-prone PCR, to obtain a mutant strain with enzyme activity increased by 5 folds. Pick up the mutant strain with maximum enzyme activity and extract plasmids for sequencing. The results show that: The gene sequence of the

mutant of N-Acetyl-D-Mannosamine Kinase is shown as SEQ ID No.26, and the corresponding amino acid is shown as SEQ ID No.27. By sequence alignment with the gene sequence of wild type N-Acetyl-D-Mannosamine Kinase, 4 base point mutations occur in total: 107A/G, 309T/G, 669G/C, and 783A/G; There occur 3 missense mutations of amino acids, of which the mutation points are: Q36R (lysine at Site 36 is replaced by arginine), I103M (isoleucine at Site 103 is replaced by methionine), and R223S (arginine at Site 223 is replaced by serine) The mutant gene is named as nanKM.

4. Integration of pTrc-nanK Gene Cassette into the nagE Gene Site in the Chromosome of Escherichia Coli

Using the nagE gene site as integration site of pTrc-nanKM gene cassette into the chromosome. To achieve integration of pTrc-nanKM gene cassette into the chromosome of Escherichia coli, first amplify the nanK segment (i.e. pTrc-nanKM) of Trc promoter, as well as the kanamycin resistance gene segment with FLP recognition site (FRT site) at its both ends: FRT-Kanr-FRT (fKanrf), and splice them. Then design homologous primers for deletion of the nagE gene sequence, and using the spliced segment of pTrc-nanKM and fKanrf as template, amplify the linear DNA full-length segment for targeting of Red recombination.

The specific operating process is provided below:

(1) PCR Amplification of pTrc-nanKM Segment

Template: nanKM/pTrc99A.

Primer Design: Forward primer (Trcff-F) SEQ ID No.20, and reverse primer (Trcff-R) SEQ ID No.21.

PCR reaction conditions: Step 1: Denature at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Produce Size: 1.05kb.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

(2) fKanrf segment amplified by PCR

Primer Design: Forward primer (mfKanf-F) SEQ ID No.1, and reverse primer (mfKanf-R) SEQ ID No.2.

Template: pPic9K.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

fKanrf size: 1.28kb. Its nucleotide sequence is SEQ ID No.3.

5 The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

(3) Amplification of fKanrf spliced with pTrc-nanKM

Primer Design: Forward primer (fKanf-F) SEQ ID No.22, and reverse primer (fKanf-R) SEQ ID No.23.

10 Template: fKanrf.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

fKanrf size from secondary amplificatino: 1.3kb.

15 The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

(4) Preparation of Linear DNA Full-length PCR Segment for Targeting of Red Recombination

20 Design of Homologous Arm Primers: Design again homologous arm primers for deletion of the nagE gene sequence: Forward primer (nagEKO-F2) SEQ ID No.24, and reverse primer (nagEKO-R2) SEQ ID No.25.

Template: Mix pTrc-nanKM PCR segment, and fKanrf PCR segment from secondary amplification in the ratio of 1:1.

25 PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product: Homologous Arm + pTrc-nanKM-fKanrf + Homologous Arm

30 The PCR product is separated by agarose gel electrophoresis, and purified and recovered to obtain 100 ng/μL linear DNA full-length PCR segment for targeting of Red recombination.

(5) Red Recombination Operation

First, pKD46 carrier is introduced into the AT-004-02 strain of *Escherichia coli*. Then, the prepared linear DNA segment for targeting is electrotransformed, and positive clones are screened. Finally, the resistance gene is removed.

1) Transformation of pKD46 Plasmid

5 ①Preparation of Competence: First, inoculate the bacterial suspension of *Escherichia coli* AT-004-02 stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h. Then add the culture solution to a 10-mL centrifuge tube, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL 0.1M CaCl₂ on an ice bath for 5
10 min. Finally, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL of 0.1M CaCl₂ on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation.

②Plasmid Transformation: Transfer 250 µL of spontaneously sedimented thalli, add 5 µL of pKD46 plasmid, and cultivate at -4°C for 30min. Then heat
15 on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h. Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate. Cultivate overnight (for 12-16 h) at 30°C. Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification. Store the positive strain for use.

20 2) Electrotransform linear DNA segment for targeting, and screen positive clone

①Preparation of Electrotransformed Competence: Inoculate the AT-004-02 strain of *Escherichia coli*, containing pKD46, into a test tube of LB medium containing Ampicillin (Amp), and shake-cultivate at 250 rpm, on the next day, inoculate in a ratio of 1% into LB medium containing Amp, and cultivate at
25 30°C; when OD₆₀₀ reaches approximately 0.2, add 0.2% L-Arabinose, and induce at 30°C for 35 min until OD₆₀₀ reaches approximately 0.4. Cool on an ice bath. Wash once with ultrapure water, wash twice with 10% glycerin, and finally resuspend with 10% glycerin; the amount of glycerin used is to produce a final concentration of the thalli concentrated by 500-1000 folds.

30 ②Transformation by electric shock: Take out a 2-mm electrotransformation cup from 70% ethanol, wash with sterilized ultrapure water, and irradiate by

UV lamp for 30 min. Precool at 4°C for 30 min. Transfer 90 µL of finally resuspended cells to a precooled centrifuge tube, add 5 µL (more than 100 ng) of the full-length PCR segment (linear DNA) obtained in Step (4), suction gently by a gun, and maintain on an ice bath for 30 min. Electrotransformation
5 Parameters: 2500V, 200Ω, 25µF.

③Resuscitate and screen positive clones: add 1 mL of LB broth medium, and cultivate at 37°C and 100rpm for 1 h. Then smear one kanamycin (Kan) plate with every 200 µL, 5 plates in total. Smear evenly and allow to air-dry. Cultivate at 30°C for 24 h. Pick up clones grown under kanamycin resistance, and carry
10 out PCR identification to screen positive clones.

No. of the obtained strain: AT-007-01 (AT-004-02, Δ nagE::pTrc-nanKM-fKanrf).

(6) Removal of the Resistance Gene

Introduce pCP20 into the above kanamycin-resistant clones, cultivate at 30°C
15 for 8 h, then increase to 42°C and cultivate overnight, and thermally induce to express FLP recombinase; the plasmids are lost gradually. Streak the plate of antibiotics-free culture medium by an inoculating loop dipped in the bacterial suspension, pick up grown monoclonal clones and dot on the kanamycin-resistant plate; those that cannot grow are clones of which the kanamycin resistance gene has been
20 removed by FLP recombination. Carry out PCR with identification primer to identify clones losing kanamycin resistance.

No. of the obtained strain: AT-007-02 (AT-004-02, Δ nagE::pTrc-nanKM).

5. Influence of pTrc-nanKM gene cassette integration on the output of N-Acetyl-D-Glucosamine

25 Carry out a shake-flask fermentation trial with the recombinant strain AT-007-02 of which the nagE gene site in the chromosome is integrated with pTrc-nanKM gene cassette, and the reference strain. Transfer the monoclonal strain freshly cultivated in culture medium of the LB plate, inoculate into a test tube (13×150mm) containing 3 mL of the LB broth culture medium, and shake-cultivate at
30 30°C and 225rpm for 8 h. Then transfer the seed culture solution, inoculate in 3% into a 250-mL shake-flask containing 50 mL of the fermentation culture solution (M9 culture solution). The initial OD600 is approximately 0.5; shake-cultivate at 37°C

and 225rpm. The fermentation cycle is 72 h. At 24 h and 48 h, adjust to pH 7.0 with 10M NaOH. According to glucose consumption in the fermentation broth, add 65% glucose solution to maintain a glucose concentration of 20 g/L. After completion of fermentation, transfer 1 mL of the fermentation broth and centrifugate. Measure the content of N-Acetyl-D-Glucosamine by HPLC method.

See Table 5 for the output from shake-flask fermentation. The results show that: The output by the reference strain AT-005-02 is very low and is not detected, while the output by the recombinant strain AT-007-02 integrated with pTrc-nanKM gene cassette is increased obviously, and is also increased significantly than that by the unmutant reference strain (AT-006-02).

Table 5, Output from Shake-flask Fermentation by the Recombinant Strain integrated with pTrc-nanKM Gene Cassette

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-005-02 (AT-004-02, Δ nagE) (Reference)	Not detected
AT-006-02 (AT-004-02, Δ nagE::pTrc-nanK)	4.5±0.4
AT-007-02 (AT-004-02, Δ nagE::pTrc-nanKM)	11.2±1.2

The above results show that: The output of N-Acetyl-D-Glucosamine may be increased obviously by overexpression of N-Acetyl-D-Mannosamine Kinase; moreover, the output of N-Acetyl-D-Glucosamine may also be greatly by the mutant screened by error-prone PCR technology, due to increased activities of the obtained mutant of the enzyme.

Example 2.c

This implementation example describes Escherichia coli strains integrated with pTrc-nanKM gene cassette, and the gene vhb thereof and its mutant for expression of vitreoscilla hemoglobin (Vhb) as well as influence on the output of N-Acetyl-D-Glucosamine.

Amplify the gene vhb for vitreoscilla hemoglobin (Vhb), insert into pTrc99A, so that vhb is controlled by Trc promoter to transform the strains, or screen a mutant of the gene vhb for vitreoscilla hemoglobin (Vhb) and insert into pTrc99A to transform the strains, to increase the microorganism's utilization ability for dissolved oxygen and increase the fermentation production the output of N-Acetyl-Glucosamine.

1. Escherichia coli strains integrated with pTrc-nanKM gene cassette, and

the gene vhb for expression of vitreoscilla hemoglobin (Vhb)

(1) Amplify the vhb Gene and Insert into pTrc99A

The nucleotide sequence the gene encoding vitreoscilla hemoglobin (Vhb) is SEQ ID No.60, and the amino acid sequence is SEQ ID No.61. Based on the preference cocon bases of *Escherichia coli*, optimize and synthesize the gene for vitreoscilla hemoglobin (Vhb), and load into pUC57 carrier. Obtain a carrier named as: pVS/pUC57.

Primer Design: Forward primer (vhb-F) SEQ ID No.62, and reverse primer (vhb-R) SEQ ID No.63.

Template: pVS/pUC57.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product Size: 441bp.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

Digest enzymatically the obtained PCR amplification segment and carrier pTrc99A with *Nco I* and *Hind III*, separate by agarose gel electrophoresis, purify and recover vhb segment and pTrc99A segment, ligate overnight with T4 DNA ligase at 16°C, and carry out identification to obtain vhb/pTrc99A plasmid.

(2) Transform *Escherichia coli* strains integrated with pTrc-NanKM gene gassette by vhb/pTrc99A

1) Preparation of Competence

①Inoculate the bacterial suspension of AT-007-02 stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h.

②Add the culture solution to a 10-mL centrifuge tube, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL 0.1M CaCl₂ on an ice bath for 5 min.

③Centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL of 0.1M CaCl₂ on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation.

2) Plasmid Transformation

①Transfer 250 μ L of spontaneously sedimentated thalli, add 5 μ L of vhb/pTrc99A plasmid, and cultivate at -4°C for 30min.

②Heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h.

③Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate.

④Cultivate overnight (for 12-16 h) at 30°C.

⑤Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification.

⑥Positive clones are stored for use.

No. of the obtained strain: AT-052 (AT-007-02, vhb/pTrc99A).

2. Escherichia coli strains integrated with pTrc-nanKM gene cassette, and the gene vhb mutant for expression of vitreoscilla hemoglobin (Vhb)

To further increase synthetic quantity of N-Acetyl-D-Glucosamine by the production strain, screen a gene mutant encoding vitreoscilla hemoglobin (Vhb) with increased activities. To achieve the purpose, amplify the cloned gene by error-prone PCR technology; used for amplified DNA polymerase, amplify the said gene under conditions leading to high-frequency mismatch, so as to obtain a high-frequency mutation in PCR products.

The specific operating process is provided below:

(1) Error-prone PCR amplification of the gene vhb for vitreoscilla hemoglobin (Vhb) in Escherichia coli

By means of Taq DNA polymerase without the property of 3' -5' proofreading function, control the frequency of random mutation under high magnesium ion concentration (8 mmol/L) and different dNTP concentrations (where, the concentration of dATP and dGTP is 1.5 mmol/L; and the concentration of dTTP and dCTP is 3.0 mmol/L), introduce random mutations into the target gene, and construct a mutant library; the template concentration A260 value is 1000 ng/mL, the enzyme concentration is 5 U/ μ L, and the primer concentration is 100 μ M.

Error-prone PCR reaction system (50 μ L): 10 $\times$ PCR reaction buffer 5 μ L, dNTP (2.5 mM) 5 μ L, MgCl₂ (25 mM) 5 μ L, Forward primer (vhb-F, SEQ ID No.62) 1 μ L,

reverse primer (vhb-R, SEQ ID No.63) 1 μ L, DNA template (nanK/pUC57) 0.1 μ L, Taq DNA polymerase 0.5 μ L, and ddH₂O 32.4 μ L.

PCR procedure: Predegenerate at 96°C for 4 min; degenerate at 94°C for 1 min, anneal at 56°C for 1 min, extend at 75°C for 2 min, and repeat for 45 cycles; finally extend at 75°C for 15 min, recover PCR product (product size: 0.44kb) by gel recovery method; transfer 5 μ L of the product and carry out 1% agarose gel electrophoresis test; the product is store at -20°C for use.

(2) Construction of the gene mutant library for vitreoscilla hemoglobin (Vhb)

Digest the above PCR product by two enzymes of restriction endonuclease *Nco I* and *Hind III*, carry out a ligation reaction with pTrc99A digested by *Nco I* and *Hind III*, then transform *Escherichia coli* AT-005-02 with the mixture of the ligated products to obtain a large amount of cloned transformants, and construct a mutant library of transformed thalli.

(3) Screening for mutants with higher activities

Using the wild type vhb/pTrc99A (AT-005-02) as reference, pick up randomly 420 mutant clones from the mutant library of transformed thalli, inoculate into 5 mL of LB medium containing 50 μ g/mL Ampicillin (Amp), shake-cultivate at 37°C and 150 rpm for 18 h, and then centrifugate at 10000 rpm for 5 min and collect thalli. Discard the supernatant, then resuspend at 4°C in 1 mL of PBS solution (pH 7.5, 10 mmol/L), carry out ultrasonicate at a voltage of 300 V for 10 min (ultrasonicate for 3 s and pause for 6 s), centrifugate, transfer the supernatant as crude extract, and carry out a method for determination of activity.

Determination of vitreoscilla hemoglobin activities: Carry out the CO-differential chromatography for determination of vitreoscilla hemoglobin activities. As the conjugate of CO and vitreoscilla hemoglobin may produce a strong absorption peak at the wavelength of 420 nm, and form a typical characteristic curve of Vhb, so the activities of Vhb may be reflected by test results of the absorption peak intensity. Test Method: Based on the feed-batch fermentation trial of different batches of the above different types of recombinant *Escherichia coli* under limited oxygen cultivation and low oxygen (dissolved oxygen $\leq$ 20%) conditions, transfer 6 mL each of the culture solution, and centrifugate and collect thalli. Wash the precipitate once with saline solution (100 mmol/L Tris-HCl, 50 mmol/L NaCl, pH7.5), then resuspend in 3 mL of the buffer, ultrasonicate, and centrifugate at 4□

and 10 000 rpm for 15 min. Transfer the supernatant, dilute by 2 folds with 3 mL of the buffer, and add Sodium Hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$) to a final concentration of 2.5 mg/mL, and then introduce CO gas into the solution. After 3 min later, scan by the ultraviolet-visible spectrometer at the wave band of 400-500 nm, and determine vitreoscilla hemoglobin activities from the maximum absorption peak (Abs) of the ultraviolet spectrum.

Screen to obtain one mutant strain with the maximum activities. Pick up the strain and extract plasmid, and carry out sequencing. The results show that: The mutant gene sequence of vitreoscilla hemoglobin is shown as SEQ ID No.64, and the corresponding amino acid sequence is shown as SEQ ID No.65. By sequence alignment with the gene sequence of the wild type N-Acetyl-D-Mannosamine Kinase, 3 base point mutations occur in total: 133A/C, 256T/G, and 284A/C. There occur 3 missense mutations of amino acids, of which the mutation points are: M45L (methionine at Site 45 is replaced by leucine), C86G (cysteine at Site 86 is replaced by glycine), and Y95S (tyrosine at Site 95 is replaced by serine). The mutant gene is named as vhbM.

(4) Transform *Escherichia coli* strains integrated with pTrc-NanKM gene cassette by vhbM/pTrc99A

1) Preparation of Competence

①Inoculate the bacterial suspension of AT-007-02 stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h.

②Add the culture solution to a 10-mL centrifuge tube, centrifugate at $4000g \times 5\text{min}$, discard the supernatant, and suspend with 5 mL 0.1M CaCl_2 on an ice bath for 5 min.

③Centrifugate at $4000g \times 5\text{min}$, discard the supernatant, and suspend with 5 mL of 0.1M CaCl_2 on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation.

2) Plasmid Transformation

①Transfer 250 μL of spontaneously sedimentated thalli, add 5 μL of vhbM/pTrc99A plasmid, and cultivate at -4°C for 30min.

②Heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium,

and shake at 30°C for 2 h.

③Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate.

④Cultivate overnight (for 12-16 h) at 30°C.

5 ⑤Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification.

⑥Positive clones are stored for use.

No. of the obtained strain: AT-053 (AT-007-02, vhbM/pTrc99A).

10 2. *Escherichia coli* strains integrated with pTrc-nanKM gene cassette, and the gene vhb thereof and its mutants for expression of vitreoscilla hemoglobin (Vhb) as well as influence on the output of N-Acetyl-D-Glucosamine

Carry out a shake-flask fermentation trial with the strains integrated with pTrc-nanKM gene cassette, and the mutant strains AT-052 and AT-053 with the gene vhb for expression of vitreoscilla hemoglobin (Vhb). Transfer the monoclonal strain freshly cultivated in culture medium of the LB plate, inoculate into a test tube (13×150mm) containing 3 mL of the LB broth culture medium, and shake-cultivate at 15 30°C and 225rpm for 8 h. Then transfer the seed culture solution, inoculate in 3% into a 250-mL shake-flask containing 50 mL of M9 culture solution. The initial OD₆₀₀ is approximately 0.5; shake-cultivate at 37°C and 225rpm. The fermentation cycle is 20 72 h. At 24 h and 48 h, adjust to pH 7.0 with 10M NaOH. According to glucose consumption in the fermentation broth, add 65% glucose solution to maintain a glucose concentration of 20 g/L. After completion of fermentation, transfer 1 mL of the fermentation broth and centrifugate. Measure the content of N-Acetyl-D-Glucosamine by HPLC method.

25 See Table 6 for the output from shake-flask fermentation by recombinant strains. The results show that: The output may be increased obviously from expression of vhb by the recombinant strains both with transformed vhb/ pTrc99A and with transformed vhbM/ pTrc99A plasmid; moreover, the output is increased more significant by the recombinant strain with with transformed vhbM/ pTrc99A plasmid.

30 Table 6. Output from Shake-flask Fermentation by the Recombinant Strains for Expression of vhb and vhbM

species	Output of
---------	-----------

	N-Acetyl-D-Glucosamine (g/L)
AT-007-02 (Reference)	11.4±1.2
AT-052 (AT-007-02, vhb/pTrc99A)	16.5±1.1
AT-053 (AT-007-02, vhbM/pTrc99A)	20.7±1.5

Example 3.a

This example describes gene nanE cloning of N-Acetyl-D-Mannosamine-6-P Epimerase (NanE), and transformed nanK/pTrc99A plasmids in *Escherichia coli*, as well as integration of ptrc- nanE gene cassette into the chromosome of *Escherichia coli*.

1. nanE Gene Cloning, Transformation of nanE/pTrc99A plasmid in *Escherichia coli*, and its Influence on the Output of N-Acetyl-D-Glucosamine

Amplify the gene nanE of *Escherichia coli* NanE (N-Acetyl-D- Mannosamine-6-P Epimerase), insert into pTrc so that nanE is controlled by Trc promoter to transform the strains for overexpression, to strengthen transformation of N-Acetyl-D-Mannosamine-6-Phosphate (ManNAc-6-P) into N-Acetyl-D-Glucosamine-6-Phosphate (GlcNAc-6-P).

1) nanE Gene Cloning of *Escherichia coli*

From NCBI, look up U00096, to obtain the nucleotide sequence SEQ ID No.28 of the nanE gene of *Escherichia coli*, which amino acid sequence is SEQ ID No.29.

Primer Design: Forward primer (nanE-F) SEQ ID No.30, and reverse primer (nanE-R) SEQ ID No.31.

Template: AT-001 (*Escherichia coli* ATCC 27325) Genome.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product Size: 690kb.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

Ligate the obtained PCR amplification segment with pUC57-T carrier, and carry out sequencing for identification to obtain nanE/ pUC57.

2) Construction and Transformation of Plasmid in which nanE gene is controlled by Trc promoter

①Plasmid Construction: Amplify plasmid nanE/ pUC57, digest enzymatically nanE/pUC57 and carrier pTrc99A with *Nco I* and *Hind III*, separate by agarose gel electrophoresis, purify to recover nanE segment and pTrc99A segment, ligate overnight with T4 DNA ligase at 16°C, and carry out identification to obtain nanE/pTrc99A plasmid.

②Preparation of Competence: First, inoculate the bacterial suspension of AT-005-02 stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h. then add the culture solution to a 10-mL centrifuge tube, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL 0.1M CaCl₂ on an ice bath for 5 min. Finally, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL of 0.1M CaCl₂ on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation.

③Plasmid Transformation: Transfer 250 µL of spontaneously sedimented thalli, add 5 µL of nanE/pTrc99A plasmid, and cultivate at -4°C for 30min. Then heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h. Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate. Cultivate overnight (for 12-16 h) at 30°C. Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification. Store the positive strain for use. Obtain Recombinant Strain nanE/pTrc99A (AT-005-02)

3) Influence of nanE/pTrc99A plasmid transformation on the output of N-Acetyl-D-Glucosamine

Carry out a shake-flask fermentation trial with the recombinant strain nanE/pTrc99A (AT-005-02) and the reference strain. Transfer the monoclonal strain freshly cultivated in culture medium of the LB plate, inoculate into a test tube (13×150mm) containing 3 mL of the LB broth culture medium, and shake-cultivate at 30°C and 225rpm for 8 h. Components of LB Broth Medium: 5 g/L yeast powder, 10 g/L peptone, and 10 g/L NaCl. Then transfer the seed culture solution, inoculate in 3% into a 250-mL shake-flask containing 50 mL of the fermentation culture solution (M9 culture solution). The initial OD₆₀₀ is approximately 0.5; shake-cultivate at 37°C and

225rpm. The fermentation cycle is 72 h. At 24 h and 48 h, adjust to pH 7.0 with 10M NaOH. According to glucose consumption in the fermentation broth, add 65% glucose solution to maintain a glucose concentration of 20 g/L. After completion of fermentation, transfer 1 mL of the fermentation broth and centrifugate. Measure the content of N-Acetyl-D-Glucosamine by HPLC method.

See Table 7 for the output from shake-flask fermentation. The results show that: The output by the reference strain AT-005-02 is very low and is not detected, while the output from overexpression by nanE gene of nanE/pTrc99A (AT-005-02), controlled by Trc promoter, is increased obviously.

Table 7. Output from Shake-flask Fermentation by the Recombinant Strain nanE/pTrc99A (AT-005-02)

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-005-02 (AT-004-02, Δ nagE) (Reference)	Not detected
nanE/pTrc99A (AT-005-02)	1.4±0.4

2. Integration of pTrc-nanE Gene Cassette into the Chromosome of Escherichia Coli

Using the nagE gene site as integration site of pTrc-nanE gene cassette into the chromosome. To achieve integration of pTrc-nanE gene cassette into the chromosome of Escherichia coli, first amplify the nanE segment (i.e. pTrc-nanE) with Trc promoter, as well as the kanamycin resistance gene segment with FLP recognition site (FRT site) at its both ends: FRT-Kanr-FRT (fKanrf), and splice them. Then design homologous primers for deletion of the nagE gene sequence, and using the spliced segment of pTrc-nanE and fKanrf as template, amplify the linear DNA full-length segment for targeting of Red recombination.

The specific operating process is provided below:

(1) PCR Amplification of pTrc-nanE Segment

Template: nanE/pTrc99A.

Primer Design: Forward primer (Trcff-F) SEQ ID No.20, and reverse primer (Trcff-R) SEQ ID No.21.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3:

Extend at 72°C for 10 min.

Product Size: 0.86kb.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

5 (2) fKanrf segment amplified by PCR

Primer Design: Forward primer (mfKanf-F) SEQ ID No.1, and reverse primer (mfKanf-R) SEQ ID No.2.

Template: pPic9K.

10 PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

fKanrf size: 1.28kb. Its nucleotide sequence is SEQ ID No.3.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

15 (3) Amplification of fKanrf spliced with pTrc-nanE

Primer Design: Forward primer (fKanf-F) SEQ ID No.22, and reverse primer (fKanf-R) SEQ ID No.23.

Template: fKanrf.

20 PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

fKanrf size from secondary amplificatino: 1.3kb.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

25 (4) Preparation of Linear DNA Full-length PCR Segment for Targeting of Red Recombination

Design of Homologous Arm Primers: Design again homologous arm primers for deletion of the nagE gene sequence: Forward primer (nagEKO-F2) SEQ ID No.24, and reverse primer (nagEKO-R2) SEQ ID No.25.

30 Template: Mix pTrc-nanE PCR segment, and fKanrf PCR segment from secondary amplification in the ratio of 1:1.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate

at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product: Homologous Arm + pTrc-nanE-fKanrf + Homologous Arm

5 The PCR product is separated by agarose gel electrophoresis, and purified and recovered to obtain 100 ng/μL linear DNA full-length PCR segment for targeting of Red recombination.

(5) Red Recombination Operation

10 First, pKD46 carrier is introduced into the AT-004-02 strain of *Escherichia coli*. Then, the prepared linear DNA segment for targeting is electrotransformed, and positive clones are screened. Finally, the resistance gene is removed.

1) Transformation of pKD46 Plasmid

15 ① Preparation of Competence: First, inoculate the bacterial suspension of *Escherichia coli* AT-004-02 stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h. then add the culture solution to a 10-mL centrifuge tube, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL 0.1M CaCl₂ on an ice bath for 5 min. Finally, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL of 0.1M CaCl₂ on an ice bath. Allow to stand at -4°C for 12 h for
20 spontaneous sedimentation.

25 ② Plasmid Transformation: Transfer 250 μL of spontaneously sedimented thalli, add 5 μL of pKD46 plasmid, and cultivate at -4°C for 30min. Then heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h. Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate. Cultivate overnight (for 12-16 h) at 30°C. Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification. Store the positive strain for use.

2) Electrotransform linear DNA segment for targeting, and screen positive clone

30 ① Preparation of Electrotransformed Competence: Inoculate the AT-004-02 strain of *Escherichia coli*, containing pKD46, into a test tube of LB medium containing Ampicillin (Amp), and shake-cultivate at 250 rpm, on the next day,

inoculate in a ratio of 1% into LB medium containing Amp, and cultivate at 30°C; when OD600 reaches approximately 0.2, add 0.2% L-Arabinose, and induce at 30°C for 35 min until OD600 reaches approximately 0.4. Cool on an ice bath. Wash once with ultrapure water, wash twice with 10% glycerin, and finally resuspend with 10% glycerin; the amount of glycerin used is to produce a final concentration of the thalli concentrated by 500-1000 folds.

②Transformation by electric shock: Take out a 2-mm electrotransformation cup from 70% ethanol, wash with sterilized ultrapure water, and irradiate by UV lamp for 30 min. Precool at 4°C for 30 min. Transfer 90 µL of finally resuspended cells to a precooled centrifuge tube, add 5 µL (more than 100 ng) of the full-length PCR segment (linear DNA) obtained in Step (4), suction gently by a gun, and maintain on an ice bath for 30 min. Electrotransformation Parameters: 2500V, 200Ω, 25µF.

③Resuscitate and screen positive clones: add 1 mL of LB broth medium, and cultivate at 37°C and 100rpm for 1 h. Then smear one kanamycin (Kan) plate with every 200 µL, 5 plates in total. Smear evenly and allow to air-dry. Cultivate at 30°C for 24 h. Pick up clones grown under kanamycin resistance, and carry out PCR identification to screen positive clones.

No. of the obtained strain: AT-030-01 (AT-004-02, $\Delta_{nagE}::pTrc-nanE-fKanrf$).

(6)Removal of the Resistance Gene

Introduce pCP20 into the above kanamycin-resistant clones, cultivate at 30°C for 8 h, then increase to 42°C and cultivate overnight, and thermally induce to express FLP recombinase; the plasmids are lost gradually. Streak the plate of antibiotics-free culture medium by an inoculating loop dipped in the bacterial suspension, pick up grown monoclonal clones and dot on the kanamycin-resistant plate; those that cannot grow are clones of which the kanamycin resistance gene has been removed by FLP recombination. Carry out PCR with identification primer to identify clones losing kanamycin resistance.

No. of the obtained strain: AT-030-02 (AT-004-02, $\Delta_{nagE}::pTrc-nanE$).

3. Influence of the integration of pTrc-nanE gene cassette on the output of

N-Acetyl-D-Glucosamine

Carry out a shake-flask fermentation trial with the recombinant strain AT-030-02 of which the nagE gene site in the chromosome is integrated with pTrc-nanE gene cassette, and the reference strain. Transfer the monoclonal strain freshly cultivated in culture medium of the LB plate, inoculate into a test tube (13×150mm) containing 3 mL of the LB broth culture medium, and shake-cultivate at 30°C and 225rpm for 8 h. Then transfer the seed culture solution, inoculate in 3% into a 250-mL shake-flask containing 50 mL of the fermentation culture solution (M9 culture solution). The initial OD₆₀₀ is approximately 0.5; shake-cultivate at 37°C and 225rpm. The fermentation cycle is 72 h. At 24 h and 48 h, adjust to pH 7.0 with 10M NaOH. According to glucose consumption in the fermentation broth, add 65% glucose solution to maintain a glucose concentration of 20 g/L. After completion of fermentation, transfer 1 mL of the fermentation broth and centrifugate. Measure the content of N-Acetyl-D-Glucosamine by HPLC method.

See Table 8 for the output from shake-flask fermentation. The results show that: The outputs by the reference strains AT-001 and AT-005-02 are very low and are not detected, while the output by the recombinant strain integrated with pTrc-nanE gene cassette is increased obviously, and is also increased significantly than that by the recombinant strain nanE/pTrc99A (AT-005-02) not integrated with the gene cassette.

Table 8, Output from Shake-flask Fermentation by the Recombinant Strain integrated with pTrc-nanE Gene Cassette

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-001 (Reference)	Not detected
AT-005-02 (AT-004-02, Δ nagE) (Reference)	Not detected
nanE/pTrc99A (AT-005-02)	1.2±0.3
AT-030-02 (AT-004-02, Δ nagE::pTrc-nanE)	2.5±0.5

Example 3.b

This implementation example describes screening for a gene mutant of

N-Acetyl-D-Mannosamine-6-P Epimerase (NanE); the said gene encodes N-Acetyl-D-Mannosamine-6-P Epimerase (NanE) with increased activities.

To further increase synthetic quantity of N-Acetyl-D-Glucosamine by the production strain, screen a gene mutant encoding N-Acetyl-D-Mannosamine-6-P Epimerase with increased activities. To achieve the purpose, amplify the cloned gene by error-prone PCR technology; used for amplified DNA polymerase, amplify the said gene under conditions leading to high-frequency mismatch, so as to obtain a high-frequency mutation in PCR products.

The specific operating process is provided below:

1. Amplification of the Gene nanE of N-Acetyl-D-Mannosamine-6-P Epimerase in Escherichia coli by Error-prone PCR

By means of Taq DNA polymerase without the property of 3' -5' proofreading function, control the frequency of random mutation under high magnesium ion concentration (8 mmol/L) and different dNTP concentrations (where, the concentration of dATP and dGTP is 1.5 mmol/L; and the concentration of dTTP and dCTP is 3.0 mmol/L), introduce random mutations into the target gene, and construct a mutant library; the template concentration A260 value is 1000 ng/mL, the enzyme concentration is 5 U/μL, and the primer concentration is 100 μM.

Error-prone PCR reaction system (50 μL): 10×PCR reaction buffer 5μL, dNTP (2.5 mM) 5 μL, MgCl₂ (2.5 mM) 5 μL, Forward primer (nanE-F, SEQ ID No.30) 1 μL, reverse primer (nanE-R, SEQ ID No.31) 1 μL, DNA template (nanE/pUC57) 0.1 μL, Taq DNA polymerase 0.5 μL, and ddH₂O 32.4μL.

PCR procedure: Predegenerate at 96°C for 4 min; degenerate at 94°C for 1 min, anneal at 56°C for 1 min, extend at 75°C for 2 min, and repeat for 45 cycles; finally extend at 75°C for 15 min, recover PCR product (product size: 0.7 kb) by gel recovery method; transfer 5 μL of the product and carry out 1% agarose gel electrophoresis test; the product is store at -20°C for use.

2. Construction of the gene mutant library of N-Acetyl-D-Mannosamine-6-P Epimerase

Digest the above PCR product by two enzymes of restriction endonuclease *Nco I* and *Hind III*, carry out a ligation reaction with pTrc99A digested by *Nco I* and *Hind III*, then transform Escherichia coli AT-005-02 with the mixture of the ligated

products to obtain a large amount of cloned transformants, and construct a mutant library of transformed thalli.

3. Screening for Mutants with High Enzyme Activities

Using the wild type NanE/pTrc99A (AT-005-02) as reference, pick up randomly
5 350 mutant clones from the mutant library of transformed thalli, inoculate into 5 mL of LB medium containing 50 µg/mL Ampicillin (Amp), shake-cultivate at 37°C and 150 rpm for 18 h, and then centrifugate at 10000 rpm for 5 min and collect thalli. Discard the supernatant, then resuspend at 4°C in 1 mL of PBS solution (pH 7.5, 10 mmol/L), carry out ultrasonicate at a voltage of 300 V for 10 min (ultrasonicate for 3
10 s and pause for 6 s), centrifugate, transfer the supernatant as crude extract of enzyme, and carry out a method for determination of enzyme activity.

Determination of N-Acetyl-D-Mannosamine-6-P Epimerase Activity: Based on the amount of N-Acetyl-D-Mannosamine-6-Phosphate (ManNAc-6-P) transformed into N-Acetyl-D-Glucosamine-6-Phosphate (GlcNAc-6-P); that is to say, using the
15 reduced amount of N-Acetyl-D-Mannosamine-6-Phosphate as test marker. Definition of Enzyme Activity Unit: Under the enzymatic reaction conditions, the enzyme amount reduced, equivalent to 1 µmol N-Acetyl-D-Mannosamine-6-Phosphate per minute, is defined as one enzyme activity unit (IU). The specific procedure is provided as follows: First, prepare isotope-labelled ManNAc-6-P as substrate.
20 Prepare a reaction solution in a total volume of 225 µL, containing ManNAc Kinase (NanK) crude solution (containing 1-5 mg protein), 20 mM ATP disodium, 60 mM Tris-HCl, pH8.1, 20 mM MgCl₂, 5 mM ManNAc, and 50nCi [¹⁴C]ManNAc. Incubate at 37°C for 30 min. Add 350 µL of alcohol to stop the reaction. The product is eluted and lyophilized with water. Secondly, prepare a reaction solution in a total volume of
25 26.5 µL as the determination system for enzyme activity, containing 1 mM isotope-labelled ManNAc-6-P, 37 mM Tris-HCl, pH 8.0, and 19 mM MgCl₂. Incubate at 37°C for 30 min, heat the reaction solution to boil for 3 min, then add 0.1 volume of alkaline phosphatase buffer to adjust pH and 20 U of alkaline phosphatase. Incubate at 37°C for 1 h, draw a sample and add on a dry chromatography paper, and
30 presoak with 1% sodium tetraborate. The used solvent system is a mixture of ethyl acetate:isopropanol:pyridine:water (50:22:14:14). Separate radioactive compounds by paper chromatography. Measure the radioactive intensity by a liquid scintillation counter, and calculate the activity units of N-Acetyl-D-Mannosamine-6-P Epimerase,

based on the amount of ManNAc-6-P transformed into GlcNAc-6-P.

The results show that: The enzyme activity of the mutant strain with maximum activity is 72 IU/mL, and the enzyme activity of the reference control is 9.5 IU/mL. Transform NanE by error-prone PCR, to obtain a mutant strain with enzyme activity increased greatly. Pick up the mutant strain with maximum enzyme activity and extract plasmids for sequencing. The results show that: The mutant gene sequence of N-Acetyl-D-Mannosamine-6-P Epimerase is shown as SEQ ID No.56, and the corresponding amino acid sequence is shown as SEQ ID No.57. Through gene sequence alignment with the wild type of N-Acetyl-D-Mannosamine-6-P Epimerase, 3 base point mutations occur in total: 198C/T, 397T/C, and 559T/C. There occur 2 missense mutations of amino acids, of which the mutation points are: Q133R (cysteine at Site 133 is replaced by arginine), and Y187H (tyrosine at Site 187 is replaced by histidine). The mutant gene is named as nanEM.

4.Integration of pTrc-nanEM Gene Cassette into the nagE Gene Site in the Chromosome of Escherichia Coli

Using the nagE gene site as integration site of pTrc-nanEM gene cassette into the chromosome. To achieve integration of pTrc-nanEM gene cassette into the chromosome of Escherichia coli, first amplify the nanEM segment (i.e. pTrc-nanEM) of Trc promoter, as well as the kanamycin resistance gene segment with FLP recognition site (FRT site) at its both ends: FRT-Kanr-FRT (fKanrf), and splice them. Then design homologous primers for deletion of the nagE gene sequence, and using the spliced segment of pTrc-nanEM and fKanrf as template, amplify the linear DNA full-length segment for targeting of Red recombination.

The specific operating process is provided below:

(1) PCR Amplification of pTrc-nanEM Segment

Template: nanEM/pTrc99A.

Primer Design: Forward primer (Trcff-F) SEQ ID No.20, and reverse primer (Trcff-R) SEQ ID No.21.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Product Size: 0.86kb.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to

recover the segment.

(2) fKanrf segment amplified by PCR

Primer Design: Forward primer (mfKanf-F) SEQ ID No.1, and reverse primer (mfKanf-R) SEQ ID No.2.

5 Template: pPic9K.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

fKanrf size: 1.28kb. Its nucleotide sequence is SEQ ID No.3.

10 The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

(3) Amplification of fKanrf spliced with pTrc-nanEM

Primer Design: Forward primer (fKanf-F) SEQ ID No.22, and reverse primer (fKanf-R) SEQ ID No.23.

15 Template: fKanrf.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

fKanrf size from secondary amplificatino: 1.3kb.

20 The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

(4) Preparation of Linear DNA Full-length PCR Segment for Targeting of Red Recombination

25 Design of Homologous Arm Primers: Design again homologous arm primers for deletion of the nagE gene sequence: Forward primer (nagEKO-F2) SEQ ID No.24, and reverse primer (nagEKO-R2) SEQ ID No.25.

Template: Mix pTrc-nanEM PCR segment, and fKanrf PCR segment from secondary amplification in the ratio of 1:1.

30 PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product: Homologous Arm + pTrc-nanEM-fKanrf + Homologous

Arm

The PCR product is separated by agarose gel electrophoresis, and purified and recovered to obtain 100 ng/μL linear DNA full-length PCR segment for targeting of Red recombination.

5 (5) Red Recombination Operation

First, pKD46 carrier is introduced into the AT-004-02 strain of Escherichia coli. Then, the prepared linear DNA segment for targeting is electrotransformed, and positive clones are screened. Finally, the resistance gene is removed.

1) Transformation of pKD46 Plasmid

10 ①Preparation of Competence: **First, inoculate the bacterial suspension of *Escherichia coli* AT-004-02 stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h. Then add the culture solution to a 10-mL centrifuge tube, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL 0.1M CaCl₂ on an ice bath for 5**
15 **min. Finally, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL of 0.1M CaCl₂ on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation.**

20 ②Plasmid Transformation: **Transfer 250 μL of spontaneously sedimented thalli, add 5 μL of pKD46 plasmid, and cultivate at -4°C for 30min. Then heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h. Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate. Cultivate overnight (for 12-16 h) at 30°C. Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification. Store the positive strain for use.**

25 2) Electrotransform linear DNA segment for targeting, and screen positive clone

30 ①Preparation of Electrotransformed Competence: **Inoculate the AT-004-02 strain of Escherichia coli, containing pKD46, into a test tube of LB medium containing Ampicillin (Amp), and shake-cultivate at 250 rpm, on the next day, inoculate in a ratio of 1% into LB medium containing Amp, and cultivate at 30°C; when OD₆₀₀ reaches approximately 0.2, add 0.2% L-Arabinose, and induce at 30°C for 35 min until OD₆₀₀ reaches approximately 0.4. Cool on an ice bath. Wash once**

with ultrapure water, wash twice with 10% glycerin, and finally resuspend with 10% glycerin; the amount of glycerin used is to produce a final concentration of the thalli concentrated by 500-1000 folds.

② Transformation by electric shock: Take out a 2-mm electrotransformation cup from 70% ethanol, wash with sterilized ultrapure water, and irradiate by UV lamp for 30 min. Precool at 4°C for 30 min. Transfer 90 µL of finally resuspended cells to a precooled centrifuge tube, add 5 µL (more than 100 ng) of the full-length PCR segment (linear DNA) obtained in Step (4), suction gently by a gun, and maintain on an ice bath for 30 min. Electrotransformation Parameters: 2500V, 200Ω, 25µF.

③ Resuscitate and screen positive clones: add 1 mL of LB broth medium, and cultivate at 37°C and 100rpm for 1 h. Then smear one kanamycin (Kan) plate with every 200 µL, 5 plates in total. Smear evenly and allow to air-dry. Cultivate at 30°C for 24 h. Pick up clones grown under kanamycin resistance, and carry out PCR identification to screen positive clones.

No. of the obtained strain: AT-031-01 (AT-004-02, Δ nagE::pTrc-nanEM-fKanrf).

(6) Removal of the Resistance Gene

Introduce pCP20 into the above kanamycin-resistant clones, cultivate at 30°C for 8 h, then increase to 42°C and cultivate overnight, and thermally induce to express FLP recombinase; the plasmids are lost gradually. Streak the plate of antibiotics-free culture medium by an inoculating loop dipped in the bacterial suspension, pick up grown monoclonal clones and dot on the kanamycin-resistant plate; those that cannot grow are clones of which the kanamycin resistance gene has been removed by FLP recombination. Carry out PCR with identification primer to identify clones losing kanamycin resistance.

No. of the obtained strain: AT-031-02 (AT-004-02, Δ nagE::pTrc-nanEM).

5. Influence of the integration of pTrc-nanEM gene cassette on the output of N-Acetyl-D-Glucosamine

Carry out a shake-flask fermentation trial with the recombinant strain AT-031-02 of which the nagE gene site in the chromosome is integrated with pTrc-nanEM gene cassette, and the reference strain. Transfer the monoclonal strain

freshly cultivated in culture medium of the LB plate, inoculate into a test tube (13×150mm) containing 3 mL of the LB broth culture medium, and shake-cultivate at 30°C and 225rpm for 8 h. Then transfer the seed culture solution, inoculate in 3% into a 250-mL shake-flask containing 50 mL of the fermentation culture solution (M9 culture solution). The initial OD600 is approximately 0.5; shake-cultivate at 37°C and 225rpm. The fermentation cycle is 72 h. At 24 h and 48 h, adjust to pH 7.0 with 10M NaOH. According to glucose consumption in the fermentation broth, add 65% glucose solution to maintain a glucose concentration of 20 g/L. After completion of fermentation, transfer 1 mL of the fermentation broth and centrifugate. Measure the content of N-Acetyl-D-Glucosamine by HPLC method.

See Table 9 for the output from shake-flask fermentation. The results show that: The output by the reference strain AT-005-02 is very low and is not detected, while the output by the recombinant strain AT-031-02 integrated with pTrc-nanEM gene cassette is increased obviously, and is also increased significantly than that by the unmutant reference strain AT-030-02.

Table 9, Output from Shake-flask Fermentation by the Recombinant Strain integrated with pTrc-nanEM Gene Cassette

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-005-02 (AT-004-02, Δ nagE) (Reference)	Not detected
AT-030-02 (AT-004-02, Δ nagE::pTrc-nanE)	2.6±0.5
AT-031-02 (AT-004-02, Δ nagE::pTrc-nanEM)	5.9±0.8

The above results show that: The output of N-Acetyl-D-Glucosamine may be increased obviously by overexpression of N-Acetyl-D-Mannosamine-6-P Epimerase; moreover, the output of N-Acetyl-D-Glucosamine may also be greatly by the mutant screened by error-prone PCR technology, due to increased activities of the obtained mutant of the epimerase.

Example 3.c

This implementation example describes Escherichia coli strains integrated with pTrc-nanEM gene cassette, and the gene vhb thereof and its mutant for expression of vitreoscilla hemoglobin (Vhb) as well as influence on the output of N-Acetyl-D-

Glucosamine.

Amplify the gene vhb for vitreoscilla hemoglobin (Vhb), insert into pTrc99A, so that vhb is controlled by Trc promoter to transform the strains, or screen a mutant of the gene vhb for vitreoscilla hemoglobin (Vhb) and insert into pTrc99A to transform the strains, to increase the microorganism's utilization ability for dissolved oxygen and increase the fermentation production the output of N-Acetyl-Glucosamine.

1. Escherichia coli strains integrated with pTrc-nanEM gene cassette, and the gene vhb for expression of vitreoscilla hemoglobin (Vhb)

(1) Amplify the vhb Gene and Insert into pTrc99A

The nucleotide sequence the gene encoding vitreoscilla hemoglobin (Vhb) is SEQ ID No.60, and the amino acid sequence is SEQ ID No.61. Based on the preference cocon bases of *Escherichia coli*, optimize and synthesize the gene for vitreoscilla hemoglobin (Vhb), and load into pUC57 carrier. Obtain a carrier named as: vhb/pUC57.

Primer Design: Forward primer (vhb-F) SEQ ID No.62, and reverse primer (vhb-R) SEQ ID No.63.

Template: vhb/pUC57.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product Size: 441bp.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

Digest enzymatically the obtained PCR amplification segment and carrier pTrc99A with *Nco I* and *Hind III*, separate by agarose gel electrophoresis, purify and recover vhb segment and pTrc99A segment, ligate overnight with T4 DNA ligase at 16°C, and carry out identification to obtain vhb/pTrc99A plasmid.

(2) Transform *Escherichia coli* strains integrated with pTrc-NanEM gene cassette by vhb/pTrc99A

1) Preparation of Competence

①Inoculate the bacterial suspension of AT-031-02 stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and

225 rpm for 2-3 h.

②Add the culture solution to a 10-mL centrifuge tube, centrifugate at $4000g \times 5\text{min}$, discard the supernatant, and suspend with 5 mL 0.1M CaCl_2 on an ice bath for 5 min.

5 ③Centrifugate at $4000g \times 5\text{min}$, discard the supernatant, and suspend with 5 mL of 0.1M CaCl_2 on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation.

2) Plasmid Transformation

10 ①Transfer 250 μL of spontaneously sedimentated thalli, add 5 μL of vhb/pTrc99A plasmid, and cultivate at -4°C for 30min.

②Heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h.

③Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate.

15 ④Cultivate overnight (for 12-16 h) at 30°C .

⑤Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification.

⑥Positive clones are stored for use.

No. of the obtained strain: AT-054 (AT-031-02, vhb/pTrc99A).

20

2. *Escherichia coli* strains integrated with pTrc-nanEM gene cassette, and the gene vhb mutant for expression of vitreoscilla hemoglobin (Vhb)

1) Preparation of Competence

25 ①Inoculate the bacterial suspension of AT-031-02 stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h.

②Add the culture solution to a 10-mL centrifuge tube, centrifugate at $4000g \times 5\text{min}$, discard the supernatant, and suspend with 5 mL 0.1M CaCl_2 on an ice bath for 5 min.

30 ③Centrifugate at $4000g \times 5\text{min}$, discard the supernatant, and suspend with

5 mL of 0.1M CaCl₂ on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation.

2) Plasmid Transformation

①Transfer 250 μL of spontaneously sedimentated thalli, add 5 μL of vhbM/pTrc99A plasmid, and cultivate at -4°C for 30min.

②Heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h.

③Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate.

④Cultivate overnight (for 12-16 h) at 30°C.

⑤Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification.

⑥Positive clones are stored for use.

No. of the obtained strain: AT-055 (AT-031-02, vhbM/pTrc99A).

2. Escherichia coli strains integrated with pTrc-nanEM gene cassette, and the gene vhb thereof and its mutants for expression of vitreoscilla hemoglobin (Vhb) as well as influence on the output of N-Acetyl-D-Glucosamine

Carry out a shake-flask fermentation trial with the strains integrated with pTrc-nanEM gene cassette, and the mutant strains AT-052 and AT-053 with the gene vhb for expression of vitreoscilla hemoglobin. Transfer the monoclonal strain freshly cultivated in culture medium of the LB plate, inoculate into a test tube (13×150mm) containing 3 mL of the LB broth culture medium, and shake-cultivate at 30°C for 8 h. Then transfer the seed culture solution, inoculate in 3% into a 250-mL shake-flask containing 50 mL of M9 culture solution. The initial OD₆₀₀ is approximately 0.5; shake-cultivate at 37°C and 225rpm. The fermentation cycle is 72 h. At 24 h and 48 h, adjust to pH 7.0 with 10M NaOH. According to glucose consumption in the fermentation broth, add 65% glucose solution to maintain a glucose concentration of 20 g/L. After completion of fermentation, transfer 1 mL of the fermentation broth and centrifugate. Measure the content of N-Acetyl-D-Glucosamine by HPLC method.

See Table 10 for the output from shake-flask fermentation by recombinant strains. The results show that: The output may be increased obviously from expression of vhb by the recombinant strains both with transformed vhb/ pTrc99A and with transformed vhbM/ pTrc99A plasmid; moreover, the output is increased more significant by the recombinant strain with with transformed vhbM/ pTrc99A plasmid.

Table 10. Output from Shake-flask Fermentation by the Recombinant Strains for Expression of vhb and vhbM

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-031-02 (Reference)	6.0±0.8
AT-054 (AT-031-02, vhb/pTrc99A)	17.1±1.2
AT-055 (AT-031-02, vhbM/pTrc99A)	20.7±1.3

Example 4.a

This example describes gene wecB cloning of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB), and transformed wecB/pTrc99A plasmids in Escherichia coli, as well as integration of ptrc-wecB gene cassette into the chromosome of Escherichia coli.

1. wecB Gene Cloning, Transformation of wecB/pTrc99A Plasmid in Escherichia coli, and its Influence on the Output of N-Acetyl-D-Glucosamine

The gene wecB of UDP-N-AcetylGlucosamine-2-Epimerase (WecB) is controlled by Trc promoter to transform strains for overexpression of the enzyme, to strengthen production of ManNAc (N-Acetyl-D-mannosamine, N-Acetyl-D-Mannosamine or N-Acetyl-D-Mannosamine) from UDP-GlcNAc (UDP-N-Acetyl Glucosamine, UDP-N-Acetyl-D-Glucosamine).

1) wecB Gene Cloning of Escherichia Coli

According to NCBI, look up the nucleotide sequence SEQ ID No.49 of the wecB gene of Escherichia coli, and its amino acid sequence is SEQ ID No.50.

Primer Design: Forward primer (TrcwecB-F) SEQ ID No.51, and reverse primer

(TrcwecB-R) SEQ ID No.52.

Template: AT-001 (*Escherichia coli* ATCC 27325) Genome.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3:
5 Extend at 72°C for 10 min.

Amplification Product Size: 1.13kb.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

Ligate the obtained PCR amplification segment with pUC57-T carrier, and carry
10 out sequencing for identification, to obtain wecB/ pUC57.

2) Construction and Transformation of Plasmid in which wecB gene is controlled by Trc promoter

①Plasmid Construction: **Amplify plasmid wecB/ pUC57, digest enzymatically wecB/pUC57 and carrier pTrc99A with *Nco I* and *Hind III*,
15 separate by agarose gel electrophoresis, purify to recover wecB segment and pTrc99A segment, ligate overnight with T4 DNA ligase at 16°C, and carry out identification to obtain wecB/pTrc99A plasmid.**

②Preparation of Competence: **First, inoculate the bacterial suspension of AT-005-02 stored at -20°C into 10 mL of LB broth medium in a ratio of
20 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h. then add the culture solution to a 10-mL centrifuge tube, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL 0.1M CaCl₂ on an ice bath for 5 min. Finally, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL of 0.1M CaCl₂ on an ice bath. Allow to stand at -4°C for 12 h for
25 spontaneous sedimentation.**

③Plasmid Transformation: **Transfer 250 μL of spontaneously sedimentated thalli, add 5 μL of wecB/pTrc99A plasmid, and cultivate at -4°C for 30min. Then heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h. Transfer 0.2 mL of the bacterial suspension, and smear
30 on a penicillin plate. Cultivate overnight (for 12-16 h) at 30°C. Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid**

for identification. Store the positive strain for use. Obtain Recombinant Strain wecB/pTrc99A (AT-005-02)

3) Influence of wecB/pTrc99A plasmid transformation on the output of N-Acetyl-D-Glucosamine

5 Carry out a shake-flask fermentation trial with the recombinant strain wecB/pTrc99A (AT-005-02) and the reference strain. Transfer the monoclonal strain freshly cultivated in culture medium of the LB plate, inoculate into a test tube (13×150mm) containing 3 mL of the LB broth culture medium, and shake-cultivate at 30°C and 225rpm for 8 h. Components of LB Broth Medium: 5 g/L yeast powder, 10 g/L peptone, and 10 g/L NaCl. Then transfer the seed culture solution, inoculate in 3% into a 250-mL shake-flask containing 50 mL of the fermentation culture solution (M9 culture solution). The initial OD₆₀₀ is approximately 0.5; shake-cultivate at 37°C and 225rpm. The fermentation cycle is 72 h. At 24 h and 48 h, adjust to pH 7.0 with 10M NaOH. According to glucose consumption in the fermentation broth, add 65% glucose solution to maintain a glucose concentration of 20 g/L. After completion of fermentation, transfer 1 mL of the fermentation broth and centrifugate. Measure the content of N-Acetyl-D-Glucosamine by HPLC method.

See Table 11 for the output from shake-flask fermentation. The results show that: The output by the reference strain AT-005-02 is very low and is not detected, while the output from overexpression by wecB gene of wecB/pTrc99A (AT-005-02), controlled by Trc promoter, is increased obviously.

25 Table 11. Output from Shake-flask Fermentation by the Recombinant Strain wecB/pTrc99A (AT-005-02)

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-005-02 (AT-004-02, Δ nagE) (Reference)	Not detected
wecB/pTrc99A (AT-005-02)	4.2±0.4

2.Integration of pTrc-wecB Gene Cassette into the Chromosome of Escherichia

Coli

Using the nagE gene site as integration site of pTrc-wecB gene cassette into the chromosome. To achieve integration of pTrc-wecB gene cassette into the chromosome of Escherichia coli, first amplify the wecB segment (i.e. pTrc-wecB) of Trc promoter, as well as the kanamycin resistance gene segment with FLP recognition site (FRT site) at its both ends: FRT-Kanr-FRT (fKanrf), and splice them. Then design homologous primers for deletion of the nagE gene sequence, and using the spliced segment of pTrc-wecB and fKanrf as template, amplify the linear DNA full-length segment for targeting of Red recombination.

The specific operating process is provided below:

(1)PCR Amplification of pTrc-wecB Segment

Template: wecB/pTrc99A.

Primer Design: Forward primer (Trcff-F) SEQ ID No.20, and reverse primer (Trcff-R) SEQ ID No.21.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Product Size: 1.3kb.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

(2)fKanrf segment amplified by PCR

Primer Design: Forward primer (mfKanf-F) SEQ ID No.1, and reverse primer (mfKanf-R) SEQ ID No.2.

Template: pPic9K.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

fKanrf size: 1.28kb. Its nucleotide sequence is SEQ ID No.3.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

(3)Amplification of fKanrf spliced with pTrc-wecB

Primer Design: Forward primer (fKanf-F) SEQ ID No.22, and reverse primer (fKanf-R) SEQ ID No.23.

Template: fKanrf.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

5 fKanrf size from secondary amplification: 1.3kb.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

(4)Preparation of Linear DNA Full-length PCR Segment for Targeting of Red Recombination

10 Design of Homologous Arm Primers: Design again homologous arm primers for deletion of the nagE gene sequence: Forward primer (nagEKO-F2) SEQ ID No.24, and reverse primer (nagEKO-R2) SEQ ID No.25.

Template: Mix pTrc-wecB PCR segment, and fKanrf PCR segment from secondary amplification in the ratio of 1:1.

15 PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product: Homologous Arm + pTrc-wecB-fKanrf + Homologous Arm

20 The PCR product is separated by agarose gel electrophoresis, and purified and recovered to obtain 100 ng/μL linear DNA full-length PCR segment for targeting of Red recombination.

(5) Red Recombination Operation

25 **First, pKD46 carrier is introduced into the AT-004-02 strain of Escherichia coli. Then, the prepared linear DNA segment for targeting is electrotransformed, and positive clones are screened. Finally, the resistance gene is removed.**

1) Transformation of pKD46 Plasmid

30 ①Preparation of Competence: **First, inoculate the bacterial suspension of Escherichia coli AT-004-02 stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h. then add the culture solution to a 10-mL centrifuge tube, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL 0.1M CaCl₂ on an ice bath for 5**

min. Finally, centrifugate at $4000g \times 5\text{min}$, discard the supernatant, and suspend with 5 mL of 0.1M CaCl_2 on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation.

②Plasmid Transformation: Transfer 250 μL of spontaneously sedimented thalli, add 5 μL of pKD46 plasmid, and cultivate at -4°C for 30min. Then heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h. Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate. Cultivate overnight (for 12-16 h) at 30°C . Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification. Store the positive strain for use.

2) Electrotransform linear DNA segment for targeting, and screen positive clone

①Preparation of Electrotransformed Competence: Inoculate the AT-004-02 strain of Escherichia coli, containing pKD46, into a test tube of LB medium containing Ampicillin (Amp), and shake-cultivate at 250 rpm, on the next day, inoculate in a ratio of 1% into LB medium containing Amp, and cultivate at 30°C ; when OD600 reaches approximately 0.2, add 0.2% L-Arabinose, and induce at 30°C for 35 min until OD600 reaches approximately 0.4. Cool on an ice bath. Wash once with ultrapure water, wash twice with 10% glycerin, and finally resuspend with 10% glycerin; the amount of glycerin used is to produce a final concentration of the thalli concentrated by 500-1000 folds.

②Transformation by electric shock: Take out a 2-mm electrotransformation cup from 70% ethanol, wash with sterilized ultrapure water, and irradiate by UV lamp for 30 min. Precool at 4°C for 30 min. Transfer 90 μL of finally resuspended cells to a precooled centrifuge tube, add 5 μL (more than 100 ng) of the full-length PCR segment (linear DNA) obtained in Step (4), suction gently by a gun, and maintain on an ice bath for 30 min. Electrotransformation Parameters: 2500V, 200 Ω , 25 μF .

③Resuscitate and screen positive clones: add 1 mL of LB broth medium, and cultivate at 37°C and 100rpm for 1 h. Then smear one kanamycin (Kan) plate with every 200 μL , 5 plates in total. Smear evenly and allow to air-dry. Cultivate

at 30°C for 24 h. Pick up clones grown under kanamycin resistance, and carry out PCR identification to screen positive clones.

No. of the obtained strain: AT-042-01 (AT-004-02, Δ nagE::pTrc-wecB-fKanrf).

(6) Removal of the Resistance Gene

5 Introduce pCP20 into the above kanamycin-resistant clones, cultivate at 30°C for 8 h, then increase to 42°C and cultivate overnight, and thermally induce to express FLP recombinase; the plasmids are lost gradually. Streak the plate of antibiotics-free culture medium by an inoculating loop dipped in the bacterial suspension, pick up grown monoclonal clones and dot on the kanamycin-resistant plate;
10 those that cannot grow are clones of which the kanamycin resistance gene has been removed by FLP recombination. Carry out PCR with identification primer to identify clones losing kanamycin resistance.

No. of the obtained strain: AT-042-02 (AT-004-02, Δ nagE::pTrc-wecB).

15 3. Influence of the integration of pTrc-wecB gene cassette on the output of N-Acetyl-D-Glucosamine

Carry out a shake-flask fermentation trial with the recombinant strain AT-042-02 of which the nagE gene site in the chromosome is integrated with pTrc-wecB gene cassette, and the reference strain. Transfer the monoclonal strain freshly cultivated in culture medium of the LB plate, inoculate into a test tube
20 (13×150mm) containing 3 mL of the LB broth culture medium, and shake-cultivate at 30°C and 225rpm for 8 h. Then transfer the seed culture solution, inoculate in 3% into a 250-mL shake-flask containing 50 mL of the fermentation culture solution (M9 culture solution). The initial OD₆₀₀ is approximately 0.5; shake-cultivate at 37°C and 225rpm. The fermentation cycle is 72 h. At 24 h and 48 h, adjust to pH 7.0 with 10M
25 NaOH. According to glucose consumption in the fermentation broth, add 65% glucose solution to maintain a glucose concentration of 20 g/L. After completion of fermentation, transfer 1 mL of the fermentation broth and centrifugate. Measure the content of N-Acetyl-D-Glucosamine by HPLC method.

See Table 12 for the output from shake-flask fermentation. The results show that:
30 The outputs by the reference strains AT-001 and AT-005-02 are very low and are not detected, while the output by the recombinant strain integrated with pTrc-wecB gene cassette is increased obviously, and is also increased significantly than that by the recombinant strain wecB/pTrc99A (AT-005-02) not integrated with the gene cassette.

Table 12, Output from Shake-flask Fermentation by the Recombinant Strain integrated with pTrc-wecB Gene Cassette

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-001 (Reference)	Not detected
AT-005-02 (AT-004-02, Δ nagE) (Reference)	Not detected
wecB/pTrc99A (AT-005-02)	4.1 $\pm$ 0.5
AT-042-02 (AT-004-02, Δ nagE::pTrc-wecB)	7.0 $\pm$ 0.8

Example 4.b

5 This implementation example describes screening for a gene mutant of UDP-N-AcetylGlucosamine-2-Epimerase (WecB); the said gene encodes UDP-N-AcetylGlucosamine-2-Epimerase (WecB) with increased activities.

To further increase synthetic quantity of N-Acetyl-D-Glucosamine by the production strain, screen a gene mutant encoding UDP-N-AcetylGlucosamine
10 -2-Epimerase with increased activities. To achieve the purpose, amplify the cloned gene by error-prone PCR technology; used for amplified DNA polymerase, amplify the said gene under conditions leading to high-frequency mismatch, so as to obtain a high-frequency mutation in PCR products.

The specific operating process is provided below:

15 1. Amplification of UDP-N-AcetylGlucosamine-2-Epimerase gene wecB by Error-prone PCR.

By means of Taq DNA polymerase without the property of 3' -5' proofreading function, control the frequency of random mutation under high magnesium ion concentration (8 mmol/L) and different dNTP concentrations (where, the
20 concentration of dATP and dGTP is 1.5 mmol/L; and the concentration of dTTP and dCTP is 3.0 mmol/L), introduce random mutations into the target gene, and construct a mutant library; the template concentration A260 value is 1000 ng/mL, the enzyme concentration is 5 U/ μ L, and the primer concentration is 100 μ M.

Error-prone PCR reaction system (50 μ L): 10 $\times$ PCR reaction buffer 5 μ L, dNTP
25 (2.5 mM) 5 μ L, MgCl₂ (25 mM) 5 μ L, Forward primer (TrcwecB-F, SEQ ID No.51)

1 μ L, reverse primer (TrcwecB-R, SEQ ID No.52) 1 μ L, DNA template (wecB/pUC57) 0.1 μ L, Taq DNA polymerase 0.5 μ L, and ddH₂O 32.4 μ L.

PCR procedure: Predegenerate at 96°C for 4 min; degenerate at 94°C for 1 min, anneal at 56°C for 1 min, extend at 75°C for 2 min, and repeat for 45 cycles; finally
5 extend at 75°C for 15 min, recover PCR product (product size: 1.13 kb) by gel recovery method; transfer 5 μ L of the product and carry out 1% agarose gel electrophoresis test; the product is store at -20°C for use.

2. Construction of the gene mutant library of UDP-N-Acetyl Glucosamine- 10 2-Epimerase

Digest the above PCR product by two enzymes of restriction endonuclease *Nco I* and *Hind III*, carry out a ligation reaction with pTrc99A digested by *Nco I* and *Hind III*, then transform *Escherichia coli* AT-005-02 with the mixture of the ligated products to obtain a large amount of cloned transformants, and construct a mutant
15 library of transformed thalli.

3. Screening for Mutants with High Enzyme Activities

Using the wild type WecB/pTrc99A (AT-005-02) as reference, pick up randomly 640 mutant clones from the mutant library of transformed thalli, inoculate
20 into 5 mL of LB medium containing 50 μ g/mL Ampicillin (Amp), shake-cultivate at 37°C and 150 rpm for 18 h, and then centrifugate at 10000 rpm for 5 min and collect thalli. Discard the supernatant, then resuspend at 4°C in 1 mL of PBS solution (pH 7.5, 10 mmol/L), carry out ultrasonicate at a voltage of 300 V for 10 min (ultrasonicate for 3 s and pause for 6 s), centrifugate, transfer the supernatant as crude
25 extract of enzyme, and carry out a method for determination of enzyme activity.

Determination of UDP-N-AcetylGlucosamine-2-Epimerase Activity: Based on the amount of UDP-N-Acetyl-D-Glucosamine transformed into N-Acetyl-D-Mannosamine. That is to say, the reduced amount of UDP-N-Acetyl-D-Glucosamine is used as test marker. Definition of Enzyme Activity Unit: Under the enzymatic
30 reaction conditions, the enzyme amount reduced, equivalent to 1 μ mol UDP-N-Acetyl-D-Glucosamine per minute, is defined as one enzyme activity unit (IU). The specific procedure is provided as follows: Transfer 20 mL of the reaction system as the system for determination of enzyme activity, which contains 45

mmol/L phosphate buffer (pH7.5), 10 mM MgCl₂ and 100 nCi of UDPGlcNAc, and 5 mg crude enzyme solution. Carry out the enzyme activity reaction by incubating on a water bath at 37°C for 30 min. Add alcohol to stop the reaction. Separate radioactive compounds by paper chromatography. Measure the radioactive intensity by a liquid scintillation counter. The solvent system used is a mixture of n-propanol:1M sodium acetate, pH 5.0:water (7:1:2). Calculate the activity units of UDP-N-AcetylGlucosamine-2-Epimerase, based on the amount of UDPGlcNAc transformed into ManNAc.

The results show that: The enzyme activity of the mutant strain with maximum activity is 653 IU/mL, and the enzyme activity of the reference control is 21.0 IU/mL. Transform WecB by error-prone PCR, to obtain a mutant strain with enzyme activity increased greatly. Pick up the mutant strain with maximum enzyme activity and extract plasmids for sequencing. The results show that: The mutant gene sequence of UDP-N-AcetylGlucosamine-2-Epimerase is shown as SEQ ID No.58, and the corresponding amino acid sequence is shown as SEQ ID No.59. Compared with gene sequence of the wild type UDP-N-AcetylGlucosamine-2-Epimerase, 5 base point mutations occur in total: 101G/C, 433C/G, 677G/T, 734T/G, and 1038T/C; There occur 4 missense mutations of amino acids, of which the mutation points are: C34S (cysteine at Site 34 is replaced by serine), H145D (histidine at Site 145 is replaced by aspartate), C226F (cysteine at Site 226 is replaced by phenylalanine) ,and V245G (valine at Site 245 is replaced by glycine). The mutant gene is named as wecBM.

4.Integration of pTrc-wecBM Gene Cassette into the nagE Gene Site in the Chromosome of Escherichia Coli

Using the nagE gene site as integration site of pTrc-wecBM gene cassette into the chromosome. To achieve integration of pTrc-wecBM gene cassette into the chromosome of Escherichia coli, first amplify the wecBM segment (i.e. pTrc-wecBM) of Trc promoter, as well as the kanamycin resistance gene segment with FLP recognition site (FRT site) at its both ends: FRT-Kanr-FRT (fKanrf), and splice them. Then design homologous primers for deletion of the nagE gene sequence, and using the spliced segment of pTrc-wecBM and fKanrf as template, amplify the linear DNA full-length segment for targeting of Red recombination.

The specific operating process is provided below:

(1)PCR Amplification of pTrc-wecBM Segment

Template: wecBM/pTrc99A.

Primer Design: Forward primer (Trcff-F) SEQ ID No.20, and reverse primer (Trcff-R) SEQ ID No.21.

5 PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Product Size: 1.3kb.

10 The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

(2)fKanrf segment amplified by PCR

Primer Design: Forward primer (mfKanf-F) SEQ ID No.1, and reverse primer (mfKanf-R) SEQ ID No.2.

Template: pPic9K.

15 PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

fKanrf size: 1.28kb. Its nucleotide sequence is SEQ ID No.3.

20 The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

(3)Amplification of fKanrf spliced with pTrc-wecBM

Primer Design: Forward primer (fKanf-F) SEQ ID No.22, and reverse primer (fKanf-R) SEQ ID No.23.

Template: fKanrf.

25 PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

fKanrf size from secondary amplificatino: 1.3kb.

30 The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

(4)Preparation of Linear DNA Full-length PCR Segment for Targeting of Red Recombination

Design of Homologous Arm Primers: Design again homologous arm primers for deletion of the nagE gene sequence: Forward primer (nagEKO-F2) SEQ ID No.24, and reverse primer (nagEKO-R2) SEQ ID No.25.

5 Template: Mix pTrc-wecBM PCR segment, and fKanrf PCR segment from secondary amplification in the ratio of 1:1.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

10 Amplification Product: Homologous Arm + pTrc-wecBM-fKanrf + Homologous Arm

The PCR product is separated by agarose gel electrophoresis, and purified and recovered to obtain 100 ng/μL linear DNA full-length PCR segment for targeting of Red recombination.

(5)Red Recombination Operation

15 **First, pKD46 carrier is introduced into the AT-004-02 strain of Escherichia coli. Then, the prepared linear DNA segment for targeting is electrotransformed, and positive clones are screened. Finally, the resistance gene is removed.**

1)Transformation of pKD46 Plasmid

20 ①Preparation of Competence: **First, inoculate the bacterial suspension of Escherichia coli AT-004-02 stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h. Then add the culture solution to a 10-mL centrifuge tube, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL 0.1M CaCl₂ on an ice bath for 5 min. Finally, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL of 0.1M CaCl₂ on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation.**

25 ②Plasmid Transformation: **Transfer 250 μL of spontaneously sedimented thalli, add 5 μL of pKD46 plasmid, and cultivate at -4°C for 30min. Then heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h. Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate. Cultivate overnight (for 12-16 h) at 30°C. Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for**

identification. Store the positive strain for use.

2) Electrotransform linear DNA segment for targeting, and screen positive clone

①Preparation of Electrotransformed Competence: Inoculate the AT-004-02 strain of Escherichia coli, containing pKD46, into a test tube of LB medium containing Ampicillin (Amp), and shake-cultivate at 250 rpm, on the next day, inoculate in a ratio of 1% into LB medium containing Amp, and cultivate at 30°C; when OD600 reaches approximately 0.2, add 0.2% L-Arabinose, and induce at 30°C for 35 min until OD600 reaches approximately 0.4. **Cool on an ice bath. Wash once with ultrapure water, wash twice with 10% glycerin, and finally resuspend with 10% glycerin; the amount of glycerin used is to produce a final concentration of the thalli concentrated by 500-1000 folds.**

②Transformation by electric shock: **Take out a 2-mm electrotransformation cup from 70% ethanol, wash with sterilized ultrapure water, and irradiate by UV lamp for 30 min. Precool at 4°C for 30 min. Transfer 90 μL of finally resuspended cells to a precooled centrifuge tube, add 5 μL (more than 100 ng) of the full-length PCR segment (linear DNA) obtained in Step (4), suction gently by a gun, and maintain on an ice bath for 30 min. Electrotransformation Parameters: 2500V, 200Ω, 25μF.**

③Resuscitate and screen positive clones: **add 1 mL of LB broth medium, and cultivate at 37°C and 100rpm for 1 h. Then smear one kanamycin (Kan) plate with every 200 μL, 5 plates in total. Smear evenly and allow to air-dry. Cultivate at 30°C for 24 h. Pick up clones grown under kanamycin resistance, and carry out PCR identification to screen positive clones.**

No. of the obtained strain: AT-043-01 (AT-004-02, Δ nagE::pTrc-wecBM-fKanrf).

(6)Removal of the Resistance Gene

Introduce pCP20 into the above kanamycin-resistant clones, cultivate at 30°C for 8 h, then increase to 42°C and cultivate overnight, and thermally induce to express FLP recombinase; the plasmids are lost gradually. Streak the plate of antibiotics-free culture medium by an inoculating loop dipped in the bacterial suspension, pick up grown monoclonal and dot on the kanamycin-resistant plate;

those that cannot grown are clones of which the kanamycin resistance gene has been removed by FLP recombination. Carry out PCR with identification primer to identify clones losing kanamycin resistance.

No. of the obtained strain: AT-043-02 (AT-004-02, Δ nagE::pTrc-wecBM).

5

5. Influence of the integration of pTrc-wecBM gene cassette on the output of N-Acetyl-D-Glucosamine

Carry out a shake-flask fermentation trial with the recombinant strain AT-043-02 of which the nagE gene site in the chromosome is integrated with pTrc-wecBM gene cassette, and the reference strain. Transfer the monoclonal strain freshly cultivated in culture medium of the LB plate, inoculate into a test tube (13×150mm) containing 3 mL of the LB broth culture medium, and shake-cultivate at 30°C and 225rpm for 8 h. Then transfer the seed culture solution, inoculate in 3% into a 250-mL shake-flask containing 50 mL of the fermentation culture solution (M9 culture solution). The initial OD600 is approximately 0.5; shake-cultivate at 37°C and 225rpm. The fermentation cycle is 72 h. At 24 h and 48 h, adjust to pH 7.0 with 10M NaOH. According to glucose consumption in the fermentation broth, add 65% glucose solution to maintain a glucose concentration of 20 g/L. After completion of fermentation, transfer 1 mL of the fermentation broth and centrifugate. Measure the content of N-Acetyl-D-Glucosamine by HPLC method.

See Table 13 for the output from shake-flask fermentation. The results show that: The output by the reference strain AT-005-02 is very low and is not detected, while the output by the recombinant strain AT-043-02 integrated with pTrc-wecBM gene cassette is increased obviously, and is also increased significantly than that by the unmutant reference strain AT-042-02.

Table 13, Output from Shake-flask Fermentation by the Recombinant Strain integrated with pTrc-wecBM Gene Cassette

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-005-02 (AT-004-02, Δ nagE) (Reference)	Not detected
AT-042-02 (AT-004-02, Δ nagE::pTrc-wecB)	7.1±0.8
AT-043-02 (AT-004-02, Δ nagE::pTrc-wecBM)	10.9±0.9

The above results show that: The output of N-Acetyl-D-Glucosamine may be increased obviously by overexpression of UDP-N-AcetylGlucosamine-2-Epimerase; moreover, the output of N-Acetyl-D-Glucosamine may also be greatly by the mutant screened by error-prone PCR technology, due to increased activities of the obtained mutant of the epimerase.

Example 4.c

This implementation example describes *Escherichia coli* strains integrated with wecBM gene cassette, and the gene vhb thereof and its mutant for expression of vitreoscilla hemoglobin (Vhb) as well as influence on the output of N-Acetyl-D-Glucosamine.

Amplify the gene vhb for vitreoscilla hemoglobin (Vhb), insert into pTrc99A, so that vhb is controlled by Trc promoter to transform the strains, or screen a mutant of the gene vhb for vitreoscilla hemoglobin (Vhb) and insert into pTrc99A to transform the strains, to increase the microorganism's utilization ability for dissolved oxygen and increase the fermentation production the output of N-Acetyl-Glucosamine.

1. *Escherichia coli* strains integrated with pTrc-wecBM gene cassette, and the gene vhb for expression of vitreoscilla hemoglobin (Vhb)

(1) Amplify the vhb Gene and Insert into pTrc99A

The nucleotide sequence the gene encoding vitreoscilla hemoglobin (Vhb) is SEQ ID No.60, and the amino acid sequence is SEQ ID No.61. Based on the preference cocon bases of *Escherichia coli*, optimize and synthesize the gene for vitreoscilla hemoglobin (Vhb), and load into pUC57 carrier. Obtain a carrier named as: vhb/pUC57.

Primer Design: Forward primer (vhb-F) SEQ ID No.62, and reverse primer (vhb-R) SEQ ID No.63.

Template: vhb/pUC57.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product Size: 441bp.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

Digest enzymatically the obtained PCR amplification segment and carrier pTrc99A with *Nco I* and *Hind III*, separate by agarose gel electrophoresis, purify and recover vhb segment and pTrc99A segment, ligate overnight with T4 DNA ligase at 16°C, and carry out identification to obtain vhb/pTrc99A plasmid.

(2) Transform *Escherichia coli* strains integrated with pTrc-wecBM gene cassette by vhb/pTrc99A

1) Preparation of Competence

①Inoculate the bacterial suspension of AT-043-02 stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h.

②Add the culture solution to a 10-mL centrifuge tube, centrifuge at 4000g × 5min, discard the supernatant, and suspend with 5 mL 0.1M CaCl₂ on an ice bath for 5 min.

③Centrifuge at 4000g × 5min, discard the supernatant, and suspend with 5 mL of 0.1M CaCl₂ on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation.

2) Plasmid Transformation

①Transfer 250 μL of spontaneously sedimentated thalli, add 5 μL of vhb/pTrc99A plasmid, and cultivate at -4°C for 30min.

②Heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h.

③Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate.

④Cultivate overnight (for 12-16 h) at 30°C.

⑤Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification.

⑥Positive clones are stored for use.

No. of the obtained strain: AT-056 (AT-043-02, vhb/pTrc99A).

2. *Escherichia coli* strains integrated with pTrc-wecBM gene cassette, and the gene vhb mutant for expression of vitreoscilla hemoglobin (Vhb)

1) Preparation of Competence

① Inoculate the bacterial suspension of AT-043-02 stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h.

5 ② Add the culture solution to a 10-mL centrifuge tube, centrifugate at $4000g \times 5\text{min}$, discard the supernatant, and suspend with 5 mL 0.1M CaCl_2 on an ice bath for 5 min.

10 ③ Centrifugate at $4000g \times 5\text{min}$, discard the supernatant, and suspend with 5 mL of 0.1M CaCl_2 on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation.

2) Plasmid Transformation

① Transfer 250 μL of spontaneously sedimentated thalli, add 5 μL of vhbM/pTrc99A plasmid, and cultivate at -4°C for 30min.

15 ② Heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h.

③ Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate.

④ Cultivate overnight (for 12-16 h) at 30°C .

20 ⑤ Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification.

⑥ Positive clones are stored for use.

No. of the obtained strain: AT-057 (AT-043-02, vhbM/pTrc99A).

25 2. *Escherichia coli* strains integrated with pTrc-wecBM gene cassette, and the gene vhb thereof and its mutants for expression of vitreoscilla hemoglobin (Vhb) as well as influence on the output of N-Acetyl-D-Glucosamine

30 Carry out a shake-flask fermentation trial with the strains integrated with pTrc-wecBM gene cassette, and the mutant strains AT-056 and AT-057 with the gene vhb for expression of vitreoscilla hemoglobin. Transfer the monoclonal strain freshly cultivated in culture medium of the LB plate, inoculate into a test tube ($13 \times 150\text{mm}$) containing 3 mL of the LB broth culture medium, and shake-cultivate at 30°C and 225rpm for 8 h. Then transfer the seed culture solution, inoculate in 3% into a

250-mL shake-flask containing 50 mL of M9 culture solution. The initial OD₆₀₀ is approximately 0.5; shake-cultivate at 37°C and 225rpm. The fermentation cycle is 72 h. At 24 h and 48 h, adjust to pH 7.0 with 10M NaOH. According to glucose consumption in the fermentation broth, add 65% glucose solution to maintain a glucose concentration of 20 g/L. After completion of fermentation, transfer 1 mL of the fermentation broth and centrifugate. Measure the content of N-Acetyl-D-Glucosamine by HPLC method.

See Table 14 for the output from shake-flask fermentation by recombinant strains. The results show that: The output may be increased obviously from expression of vhb by the recombinant strains both with transformed vhb/ pTrc99A and with transformed vhbM/ pTrc99A plasmid; moreover, the output is increased more significant by the recombinant strain with with transformed vhbM/ pTrc99A plasmid.

Table 14. Output from Shake-flask Fermentation by the Recombinant Strains Integrated with pTrc-wecBM Gene Cassette for Expression of vhb and vhbM

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-043-02 (AT-004-02, Δ nagE::pTrc-wecBM) (reference)	10.8±0.9
AT-056 (AT-043-02, vhb/pTrc99A)	17.3±1.4
AT-057 (AT-043-02, vhbM/pTrc99A)	21.4±1.4

Example 5.a

This implementation example describes influences of the strains on the output of N-Acetyl-D-Glucosamine, of which the natural endogenous promoter of the nagB gene is replaced by Trc promoter, or the natural endogenous promoter of the glmS gene is further deleted.

1.The natural endogenous promoter of the nagB gene is replaced by Trc promoter

The gene promoter of D-Glucosamine-6-Phosphate Deaminase (NagB) in nag regulon (nagE-nagBACD) is replaced by Trc promoter. The reaction catalyzed by D-Glucosamine-6-Phosphate Deaminase (NagB) is reversible; overexpression of nagB may accelerate the forward catalytic reaction by NagB, to achieve the purpose

of increasing D-Glucosamine-6-Phosphate (GlcN-6-P).

First, amplify Trc promoter segment and fKanrf segment, and splice together. Then design homologous primers, and amplify linear DNA full-length segment for targeting of Red recombination.

5 (1) Amplification of Trc promoter sequence

According to public information, look up Trc promoter sequence: SEQ ID No.32.

Primer Design: Forward primer (KanTrcRed-F) SEQ ID No.33, and reverse primer (KanTrcRed-R) SEQ ID No.34.

10 **Template: pTrc99A**

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Product Size: 166bp.

15 **The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.**

(2) Amplify the kanamycin resistance gene with recognition sites (FRT sites) for FLP recombinase at its both ends: fKanrf

20 **Primer Design: Forward primer (mfKanf-F) SEQ ID No.1, and reverse primer (mfKanf-R) SEQ ID No.2.**

Template: pPic9K.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

25 **fKanrf size: 1.28kb. Its nucleotide sequence is SEQ ID No.3.**

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

(3) Amplification of fKanrf spliced with Trc promoter

30 **Primer Design: Forward primer (fKanfRed-F1) SEQ ID No.35, and reverse primer (fKanfRed-R1) SEQ ID No.36.**

Template: fKanrf.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2:

Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

fKanrf size from secondary amplificatino: 1.3kb.

5 The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

(4) Preparation of Linear DNA Full-length PCR Segment for Targeting of Red Recombination

10 Design of Homologous Arm Primers: From NCBI, look up NC_000913, nagB promoter sequence and nagA gene sequence SEQ ID No.13 of *Escherichia coli* str. K-12, and design the homologous arm primers for deletion of nagB promoter: Forward primer (nagBKO-F1) SEQ ID No.40 and reverse primer (nagBKO-R1) SEQ ID No.41.

Template: Mix Trc promoter PCR segment and fKanrf PCR segment from secondary amplification in the ratio of 1:1.

15 PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product: Homologous Arm + fKanrf + Trc Promoter + Homologous Arm.

20 The PCR product is separated by agarose gel electrophoresis, and purified and recovered to obtain 100 ng/μL linear DNA full-length PCR segment for targeting of Red recombination.

(5) Red Recombination Operation

25 First, pKD46 carrier is introduced into the AT-005-02 strain of *Escherichia coli*. Then, the prepared linear DNA segment for targeting is electrotransformed, and positive clones are screened. Finally, the resistance gene is removed.

No. of the obtained strain: AT-048(AT-005-02, ΔnagB promotor::Trc promoter).

2. Deletion of the natural endogenous promoter of the glmS gene

30 Deletion of the gene promoter of Glucosamine-6-Phosphate Synthase (glmS). Glucosamine-6-Phosphate Synthase (GlmS), also called as L-Glutamine-D-fructose-6-phosphate aminotransferase, can catalyze amination of Glucose-6-Phosphate (Glc-6-P) into D-Glucosamine-6-Phosphate (GlcN-6-P), but

has a severe problem for product inhibition. When the promoter sequence is deleted so that the enzyme cannot be expressed, it may solve product inhibition of GlcN-6-P.

First, amplify fKanrf segment; then design homologous primers, and amplify linear DNA full-length segment for targeting of Red recombination.

- 5 (1) Amplify the kanamycin resistance gene with recognition sites (FRT sites) for FLP recombinase at its both ends: fKanrf

Primer Design: Forward primer (mfKanf-F) SEQ ID No.1, and reverse primer (mfKanf-R) SEQ ID No.2.

Template: pPic9K.

- 10 PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

fKanrf size: 1.28kb. Its nucleotide sequence is SEQ ID No.3.

- 15 The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

(2) Preparation of Linear DNA Full-length PCR Segment for Targeting of Red Recombination

- 20 Design of Homologous Primers: From NCBI, look up NC_000913, the gene promoter sequence SEQ ID No.42 of Glucosamine-6-Phosphate Synthase (GlmS) of *Escherichia coli* str. K-12, and design the homologous arm primers for deletion of glmS gene promoter: Forward primer (ProglmsKO-F) SEQ ID No.43 and reverse primer (ProglmsKO-R) SEQ ID No.44.

Template: fKanrf PCR Segment.

- 25 PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product: Homologous Arm + fKanf + Homologous Arm.

- 30 The PCR product is separated by agarose gel electrophoresis, and purified and recovered to obtain 100 ng/μL linear DNA full-length PCR segment for targeting of Red recombination.

(3) Red Recombination Operation

First, pKD46 carrier is introduced into the AT-048 strain of *Escherichia coli*. Then, the prepared linear DNA segment for targeting is electrotransformed, and

positive clones are screened. Finally, the resistance gene is removed.

No. of the obtained strain: AT-049(AT-048, Δ glmS promotor).

3. Influence of the strains of which nagB promoter replaced by a promoter with higher expression level, or glmS promoter is further deleted, on the output of

5 N-Acetyl-D-Glucosamine

Carry out a shake-flask fermentation trial with the strain of which nagB promoter is replaced by a promoter with higher expression level, and the recombinant strain of which glmS promoter is further deleted. Transfer the monoclonal strain freshly cultivated in culture medium of the LB plate, inoculate into a test tube (13×150mm) containing 3 mL of the LB broth culture medium, and shake-cultivate at 10 30°C and 225rpm for 8 h. Then transfer the seed culture solution, inoculate in 3% into a 250-mL shake-flask containing 50 mL of the fermentation culture solution (M9 culture solution). The initial OD600 is approximately 0.5; shake-cultivate at 37°C and 225rpm. The fermentation cycle is 72 h. At 24 h and 48 h, adjust to pH 7.0 with 15 10M NaOH. According to glucose consumption in the fermentation broth, add 65% glucose solution to maintain a glucose concentration of 20 g/L. After completion of fermentation, transfer 1 mL of the fermentation broth and centrifugate. Measure the content of N-Acetyl-D-Glucosamine by HPLC method.

See Table 15 for the output from shake-flask fermentation. The results show that: 20 The output of N-Acetyl-D-Glucosamine is obviously increased by the recombinant strains of which nagB promoter is replaced by Trc promoter, or glmS promoter is further deleted.

Table 15, Output from Shake-flask Fermentation by Recombinant Strains of which nagB promoter is replaced or glmS promoter is further deleted

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-001 (Reference)	Not detected
AT-005-02 (Reference)	Not detected
AT-048(AT-005-02, Δ nagB promotor::Trc promotor)	3.3±0.4
AT-049(AT-048, Δ glmS promotor)	8.7±0.9

Example 5.b

This implementation example describes influences of the strains on the output of N-Acetyl-D-Glucosamine, of which the natural endogenous promoter of the glmS gene is replaced by Trc promoter, and the natural endogenous promoter of the nagB gene is further deleted.

5

1.The natural endogenous promoter of the glmS gene is replaced by Trc promoter

The gene promoter sequence of L-Glutamine-D-Fructose-6-Phosphate Aminotransferase is replaced by Trc promoter sequence. L-Glutamine-6-Phosphate
10 Aminotransferase, also called as Glucosamine-6-Phosphate Synthase (GlmS); replacement of its promoter sequence is replaced by Trc promoter sequence may overexpress glmS, and accelerate GlmS catalytic function, to achieve the purpose of increasing D-Glucosamine-6-Phosphate (GlcN-6-P).

First, amplify Trc promoter sequence segment and fKanrf segment, and splice
15 together. Then design homologous primers, and amplify linear DNA full-length segment for targeting of Red recombination.

(1) Amplification of Linear DNA Full-length PCR Segment for Targeting of Red Recombination

Design of homologous arm primers: According to the glmS gene promoter
20 sequence SEQ ID No.42, design homologous arm primers with the promoter replaced by Trc promoter: Forward primer (ProglmspTrc-F) SEQ ID No.45, and reverse primer (ProglmspTrc-R) SEQ ID No.46.

Template: Mix Trc promoter PCR segment and fKanrf PCR segment from secondary amplification in the ratio of 1:1.

25 PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product: Homologous Arm + fKanrf + Trc Promoter + Homologous Arm.

30 The PCR product is separated by agarose gel electrophoresis, and purified and recovered to obtain 100 ng/μL linear DNA full-length PCR segment for targeting of Red recombination.

(2) Red Recombination Operation

First, pKD46 carrier is introduced into the AT-005-02 strain of Escherichia coli. Then, the prepared linear DNA segment for targeting is electrotransformed, and positive clones are screened. Finally, the resistance gene is removed.

No. of the obtained strain: AT-050 (AT-005-02, Δ glmS promoter::Trc promoter).

2. Deletion of the natural endogenous promoter of NagB gene

Deletion of the gene promoter sequence of D-Glucosamine-6-Phosphate Deaminase (NagB) in the nag regulon (nagE-nagBACD), so that nagB loses its function, may eliminate the reverse catalytic function of NagB and reduce production of Glc-6-P from GlcN-6-P.

First, amplify fKanrf segment; then design homologous primers, and prepare linear DNA full-length segment for targeting of Red recombination.

(1) Preparation of Linear DNA Full-length PCR Segment for Targeting of Red Recombination

Design of Homologous Arm Primers: According to nagB promoter sequence and nagA gene sequence SEQ ID No.13, design the homologous arm primers for deletion of nagB promoter sequence: Forward primer (nagBKO-F2) SEQ ID No.47 and reverse primer (nagBKO-R2) SEQ ID No.48.

Template: fKanrf PCR Segment

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product: Homologous Arm + fKanrf + Homologous Arm.

The PCR product is separated by agarose gel electrophoresis, and purified and recovered to obtain 100 ng/μL linear DNA full-length PCR segment for targeting of Red recombination.

(2) Red Recombination Operation

First, pKD46 carrier is introduced into the AT-050 strain of Escherichia coli. Then, the prepared linear DNA segment for targeting is electrotransformed, and positive clones are screened. Finally, the resistance gene is removed.

No. of the obtained strain: AT-051 (AT-050, Δ nagB promoter).

3. Influence of the strains of which glmS promoter replaced by a promoter with higher expression level, or is further deleted, on the output of N-Acetyl-D-Glucosamine

Carry out a shake-flask fermentation trial with the strain of which glmS promoter is replaced by a promoter with higher expression level, and the recombinant strain of which nagB promoter is further deleted.

Transfer the monoclonal strain freshly cultivated in culture medium of the LB plate, inoculate into a test tube (13×150mm) containing 3 mL of the LB broth culture medium, and shake-cultivate at 30°C and 225rpm for 8 h. Then transfer the seed culture solution, inoculate in 3% into a 250-mL shake-flask containing 50 mL of the fermentation culture solution (M9 culture solution). The initial OD600 is approximately 0.5; shake-cultivate at 37°C and 225rpm. The fermentation cycle is 72 h. At 24 h and 48 h, adjust to pH 7.0 with 10M NaOH. According to glucose consumption in the fermentation broth, add 65% glucose solution to maintain a glucose concentration of 20 g/L. After completion of fermentation, transfer 1 mL of the fermentation broth and centrifugate. Measure the content of N-Acetyl-D-Glucosamine by HPLC method.

See Table 16 for the output from shake-flask fermentation. The results show that: The output of N-Acetyl-D-Glucosamine is not obviously increased by the recombinant strains of which glmS promoter is replaced by Trc promoter, and is not detected. However, the output of N-Acetyl-D-Glucosamine is increased obviously when nagB is meanwhile deleted, than that by the reference strain.

Table 16, Output from Shake-flask Fermentation by Recombinant Strains of which glmS promoter is replaced or nagB promoter is further deleted

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-001 (Reference)	Not detected
AT-005-02 (Reference)	Not detected
AT-050 (AT-005-02, Δ glmS promotor::Trc promotor)	Not detected
AT-051 (AT-050, Δ nagB promotor)	5.6±0.6

Example 5.c

This implementation example describes *Escherichia coli* strains integrated of which the natural endogenous promoter of *glmS* gene and *nagB* gene is replaced and/or deleted, and the gene *vhb* thereof and its mutant for expression of vitreoscilla hemoglobin (Vhb) as well as influence on the output of N-Acetyl-D-Glucosamine.

Amplify the gene *vhb* for vitreoscilla hemoglobin (Vhb), insert into pTrc99A, so that *vhb* is controlled by Trc promoter to transform the strains, or screen a mutant of the gene *vhb* for vitreoscilla hemoglobin (Vhb) and insert into pTrc99A to transform the strains, to increase the microorganism's utilization ability for dissolved oxygen and increase the fermentation production the output of N-Acetyl-Glucosamine.

1. *Escherichia coli* strains of which the *nagB* promoter is replaced by a promoter with higher expression level and the *glmS* promoter is further deleted, and *vhb* and its mutants for expression of vitreoscilla hemoglobin (Vhb)

(1) Amplify the *vhb* Gene and Insert into pTrc99A

The nucleotide sequence the gene encoding vitreoscilla hemoglobin (Vhb) is SEQ ID No.60, and the amino acid sequence is SEQ ID No.61. Based on the preference cocon bases of *Escherichia coli*, optimize and synthesize the gene for vitreoscilla hemoglobin (Vhb), and load into pUC57 carrier. Obtain a carrier named as: *vhb/pUC57*.

Primer Design: Forward primer (*vhb-F*) SEQ ID No.62, and reverse primer (*vhb-R*) SEQ ID No.63.

Template: *vhb/pUC57*.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product Size: 441bp.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

Digest enzymatically the obtained PCR amplification segment and carrier pTrc99A with *Nco I* and *Hind III*, separate by agarose gel electrophoresis, purify and recover *vhb* segment and pTrc99A segment, ligate overnight with T4 DNA ligase at 16°C, and carry out identification to obtain *vhb/pTrc99A* plasmid.

(2) *Escherichia coli* strains of which the *nagB* promoter is transformed with

vhb/pTrc99A and vhbM/pTrc99A to produce a promoter with higher expression level, and the glmS promoter is further deleted

1) Preparation of Competence

①Inoculate the bacterial suspension of AT-049 stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h.

②Add the culture solution to a 10-mL centrifuge tube, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL 0.1M CaCl₂ on an ice bath for 5 min.

③Centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL of 0.1M CaCl₂ on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation.

2) Plasmid Transformation

①Transfer 250 µL of spontaneously sedimentated thalli, add 5 µL of vhb/pTrc99A and vhbM/pTrc99A plasmids, respectively, and cultivate at -4°C for 30min.

②Heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h.

③Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate.

④Cultivate overnight (for 12-16 h) at 30°C.

⑤Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification.

⑥Positive clones are stored for use.

No. of the obtained strains: AT-058 (AT-049, vhb/pTrc99A), and AT-059 (AT-049, vhbM/pTrc99A).

2. Escherichia coli strains of which the natural endogenous promoter of the glmS gene is replaced by Trc promoter, and the natural endogenous promoter of the nagB gene is deleted, and the gene vhb mutants for expression of vitreoscilla hemoglobin

1) Preparation of Competence

①Inoculate the bacterial suspension of AT-051 stored at -20°C into 10 mL of LB broth medium in a ratio of 1:50-100, and shake-cultivate at 37°C and 225 rpm for 2-3 h.

5 ②Add the culture solution to a 10-mL centrifuge tube, centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL 0.1M CaCl₂ on an ice bath for 5 min.

10 ③Centrifugate at 4000g × 5min, discard the supernatant, and suspend with 5 mL of 0.1M CaCl₂ on an ice bath. Allow to stand at -4°C for 12 h for spontaneous sedimentation.

2) Plasmid Transformation

①Transfer 250 μL of spontaneously sedimentated thalli, add 5 μL of vhb/pTrc99A and vhbM/pTrc99A plasmids, respectively, and cultivate at -4°C for 30min.

15 ②Heat on a water bath at 42°C for 1.5 min, add 0.7 mL of SOC medium, and shake at 30°C for 2 h.

③Transfer 0.2 mL of the bacterial suspension, and smear on a penicillin plate.

④Cultivate overnight (for 12-16 h) at 30°C.

20 ⑤Pick up monoclonal, add 5 mL of LB broth medium and cultivate, and withdraw plasmid for identification.

⑥Positive clones are stored for use.

No. of the obtained strains: AT-060 (AT-051, vhb/pTrc99A), and AT-061 (AT-051, vhbM/pTrc99A).

25

2. *Escherichia coli* strains integrated of which the natural endogenous promoter of *glmS* gene and *nagB* gene is replaced and/or deleted, and the gene *vhb* thereof and its mutant for expression of *vitreoscilla* hemoglobin (Vhb) as well as influence on the output of N-Acetyl-D-Glucosamine

30 Carry out a shake-flask fermentation trial with the strains of which the natural endogenous promoter of *glmS* gene and *nagB* gene is replaced and/or deleted, and the

strains AT-058, AT-059, AT-060, and AT-061 with the gene vhb thereof and its mutant for expression of vitreoscilla hemoglobin (Vhb). Transfer the monoclonal strain freshly cultivated in culture medium of the LB plate, inoculate into a test tube (13×150mm) containing 3 mL of the LB broth culture medium, and shake-cultivate at 30°C and 225rpm for 8 h. Then transfer the seed culture solution, inoculate in 3% into a 250-mL shake-flask containing 50 mL of M9 culture solution. The initial OD₆₀₀ is approximately 0.5; shake-cultivate at 37°C and 225rpm. The fermentation cycle is 72 h. At 24 h and 48 h, adjust to pH 7.0 with 10M NaOH. According to glucose consumption in the fermentation broth, add 65% glucose solution to maintain a glucose concentration of 20 g/L. After completion of fermentation, transfer 1 mL of the fermentation broth and centrifugate. Measure the content of N-Acetyl-D-Glucosamine by HPLC method.

See Table 17 for the output from shake-flask fermentation by recombinant strains. The results show that: The output may be increased obviously from expression of vhb by the recombinant strains both with transformed vhb/ pTrc99A and with transformed vhbM/ pTrc99A plasmid; moreover, the output is increased more significant by the recombinant strain with with transformed vhbM/ pTrc99A plasmid.

Table 17. Output from Shake-flask Fermentation by the Recombinant Strains for Expression of vhb and vhbM

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-049(AT-048, Δ glmS promotor)	8.6±0.9
AT-058 (AT-049, vhb/pTrc99A)	10.5±1.0
AT-059 (AT-049, vhbM/pTrc99A)	13.5±1.1
AT-051 (AT-050, Δ nagB promotor)	5.7±0.5
AT-060 (AT-051, vhb/pTrc99A)	8.4±0.6
AT-061 (AT-051, vhbM/pTrc99A)	11.7±1.0

Example 6

This implementation example describes Escherichia coli strains integrated with pTrc-nanKM gene cassette, of which the natural endogenous promoter of the nanE gene is replaced by Trc promoter, and the gene vhb thereof and its mutant for expression of vitreoscilla hemoglobin (Vhb) as well as influence on the output of N-Acetyl-D-Glucosamine.

1. The natural endogenous promoter with nanE gene of Escherichia coli strains integrated with pTrc-nanKM cassette is replaced with Trc promoter

First, amplify Trc promoter sequence segment and fKanrf segment, and splice together. Then design homologous primers, and amplify linear DNA full-length segment for targeting of Red recombination.

(1) Amplification of Trc promoter sequence

According to public information, look up Trc promoter sequence: SEQ ID No.32.

Primer Design: Forward primer (KanTrcRed-F) SEQ ID No.33, and reverse primer (KanTrcRed-R) SEQ ID No.34.

Template: pTrc99A

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Product Size: 166bp.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

(2) Amplify the kanamycin resistance gene with recognition sites (FRT sites) for FLP recombinase at its both ends: fKanrf

Primer Design: Forward primer (mfKanf-F) SEQ ID No.1, and reverse primer (mfKanf-R) SEQ ID No.2.

Template: pPic9K.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

fKanrf size: 1.28kb. Its nucleotide sequence is SEQ ID No.3.

The PCR product is separated by 1% agarose gel electrophoresis, and purified to

recover the segment.

(3)Amplification of fKanrf spliced with Trc promotor

Primer Design: Forward primer (fKanfRed-F1) SEQ ID No.35, and reverse primer (fKanfRed-R1) SEQ ID No.36.

5 **Template: fKanrf.**

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

fKanrf size from secondary amplificatino: 1.3kb.

10 The PCR product is separated by 1% agarose gel electrophoresis, and purified to recover the segment.

(4)Preparation of Linear DNA Full-length PCR Segment for Targeting of Red Recombination

15 **Design homologous primers: Based on promoter sequence SEQ ID No.37 of the nanE gene. Design primers to be replaced with Trc promoter: Forward primer (ProNanEpTrc-F) SEQ ID No.38, and reverse primer (ProNanEpTrc-R) SEQ ID No.39.**

Template: Mix Trc promoter PCR segment and fKanrf PCR segment from secondary amplification in the ratio of 1:1.

20 **PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.**

Amplification Product: Homologous Arm + fKanrf + Trc Promoter + Homologous Arm.

25 The PCR product is separated by agarose gel electrophoresis, and purified and recovered to obtain 100 ng/μL linear DNA full-length PCR segment for targeting of Red recombination.

(5)Red Recombination Operation

30 **First, pKD46 carrier is introduced into the AT-007-02 strain of Escherichia coli. Then, the prepared linear DNA segment for targeting is electrotransformed, and positive clones are screened. Finally, the resistance gene is removed.**

No. of the obtained strain: AT-009 (AT-007-02, ΔnanE promoter::Trc promoter).

2. Escherichia coli strains integrated with pTrc-nanEM gene cassette, of which the natural endogenous promoter of the nanE gene is replaced by Trc promoter, and the gene vhb thereof and its mutant for expression of vitreoscilla hemoglobin (Vhb)

First, prepare competence of recombinant Escherichia coli strain AT-009; then, transform vhb/pTrc99A and vhbM/pTrc99A plasmids into AT-009 by CaCl₂ transformation method, pick up monoclones and cultivate, and pipet plasmid to identify positive clones.

No. of the obtained strains: AT-062 (AT-009, vhb/pTrc99A), and AT-063 (AT-009, vhbM/pTrc99A).

Carry out a shake-flask fermentation trial with the strains AT-062 and AT-063 with the gene vhb and its mutant for expression of vitreoscilla hemoglobin. See Table 18 for the output from shake-flask fermentation by recombinant strains. The results show that: The output may be increased obviously from expression of vhb by the recombinant strains both with transformed vhb/ pTrc99A and with transformed vhbM/ pTrc99A plasmid; moreover, the output is increased more significant by the recombinant strain with with transformed vhbM/ pTrc99A plasmid.

Table 18, Output from Shake-flask Fermentation by the Recombinant Strain

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-009 (AT-007-02, Δ nanE promoter::Trc promoter) (reference)	19.9±1.5
AT-062 (AT-009, vhb/pTrc99A)	23.9±1.4
AT-063 (AT-009, vhbM/pTrc99A)	26.8±1.3

Example 7

This implementation example describes Escherichia coli strains integrated with pTrc-nanKM and influences of those on the output of N-Acetyl-D-Glucosamine, of which the natural endogenous promoter of the gene glmS of Glucosamine-6-Phosphate Synthase (GlmS) and/or the nagB gene of

D-Glucosamine-6-Phosphate Deaminase (NagB) is replaced and/or deleted, and the gene vhb and its mutants for expression of vitreoscilla hemoglobin (Vhb).

In *Escherichia coli* strains integrated with pTrc-nanKM cassette, the natural endogenous promoter of the nanB gene is replaced by Trc promoter to produce AT-010 (AT-007-02, Δ nagB promoter::Trc promoter), and the natural endogenous promoter of the glmS gene is further deleted to produce AT-011 (AT-010, Δ glmS promoter); In *Escherichia coli* strains integrated with pTrc-nanKM cassette, the natural endogenous promoter of the glmS gene is replaced by Trc promoter to produce AT-012 (AT-007-02, Δ nagB promoter::Trc promoter), and the natural endogenous promoter of the nagB gene is further deleted to produce AT-013 (AT-012, Δ nagB promoter).

Prepare competence of recombinant *Escherichia coli* strain AT-11 and AT-013; then, transform vhb/pTrc99A and vhbM/pTrc99A plasmids into AT-011 and AT-013 by CaCl_2 transformation method, pick up monoclones and cultivate, and pipet plasmid to identify positive clones.

No. of the obtained strains: AT-064 (AT-011, vhb/pTrc99A), AT-065 (AT-011, vhbM/pTrc99A), AT-066 (AT-013, vhb/pTrc99A), and AT-067 (AT-013, vhbM/pTrc99A).

Carry out a shake-flask fermentation trial with the strains AT-064, AT-065, AT-066, and AT-067 with the gene vhb and its mutant for expression of vitreoscilla hemoglobin. See Table 19 for the output from shake-flask fermentation by recombinant strains. The results show that: The output may be increased obviously from expression of vhb by the recombinant strains both with transformed vhb/pTrc99A and with transformed vhbM/pTrc99A plasmid; moreover, the output is increased more significant by the recombinant strain with with transformed vhbM/pTrc99A plasmid.

Table 19, Output from Shake-flask Fermentation by the Recombinant Strain

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-011 (AT-010, Δ glmS promoter) (reference)	21.6 $\pm$ 1.4
AT-064 (AT-011, vhb/pTrc99A)	24.8 $\pm$ 1.4
AT-065 (AT-011, vhbM/pTrc99A)	28.6 $\pm$ 1.5
AT-013 (AT-012, Δ nagB promoter) (reference)	21.3 $\pm$ 1.6

AT-066 (AT-013, vhb/pTrc99A)	24.6±1.4
AT-067 (AT-013, vhbM/pTrc99A)	27.9±1.3

Example 8

This implementation example describes *Escherichia coli* strains integrated with pTrc-nanKM gene cassette, of which the natural endogenous promoter of the *wecB* gene is replaced by Trc promoter, and the gene *vhb* thereof and its mutant for expression of vitreoscilla hemoglobin (Vhb) as well as influence on the output of N-Acetyl-D-Glucosamine.

1. The natural endogenous promoter of the *wecB* gene of *Escherichia coli* strains integrated with pTrc-nanKM cassette is replaced by Trc promoter

First, amplify Trc promoter sequence segment and fKanrf segment, and splice together. Then design homologous primers, and amplify linear DNA full-length segment for targeting of Red recombination.

(1) Preparation of Linear DNA Full-length PCR Segment for Targeting of Red Recombination

Design of Homologous Arm Primers: According to NCBI, look up NC_000913 to obtain the nucleotide sequence SEQ ID No.53 of the gene promoter of *Escherichia coli* UDP-N-AcetylGlucosamine-2-Epimerase (*WecB*), design homologous arm primers with the promoter replaced by Trc promoter: Forward primer (ProwecBpTrc-F) SEQ ID No.54, and reverse primer (Pro *wecB*pTrc-R) SEQ ID No.55.

Template: Mix Trc promoter PCR segment and fKanrf PCR segment from secondary amplification in the ratio of 1:1.

PCR reaction conditions: Step 1: Degenerate at 94°C for 1 min; Step 2: Incubate at 94°C for 30s, at 55°C for 30s, and at 72°C for 40s, and carry out 30 cycles; Step 3: Extend at 72°C for 10 min.

Amplification Product: Homologous Arm + fKanrf + Trc Promoter + Homologous Arm.

The PCR product is separated by agarose gel electrophoresis, and purified and recovered to obtain 100 ng/μL linear DNA full-length PCR segment for targeting of Red recombination.

(2) Red Recombination Operation

First, pKD46 carrier is introduced into the AT-007-02 strain of *Escherichia coli*. Then, the prepared linear DNA segment for targeting is electrotransformed, and positive clones are screened. Finally, the resistance gene is removed.

No. of the obtained strain: AT-019(AT-007-02, Δ_{wecB} promotor::Trc promoter).

2. *Escherichia coli* strains integrated with pTrc-nanEM gene cassette, of which the natural endogenous promoter of the *wecB* gene is replaced by Trc promoter, and the gene *vhb* thereof and its mutant for expression of vitreoscilla hemoglobin (Vhb)

First, prepare competence of recombinant *Escherichia coli* strain AT-019; then, transform *vhb*/pTrc99A and *vhbM*/pTrc99A plasmids into AT-019 by CaCl_2 transformation method, pick up monoclones and cultivate, and pipet plasmid to identify positive clones.

No. of the obtained strains: AT-068 (AT-019, *vhb*/pTrc99A), and AT-069 (AT-019, *vhbM*/pTrc99A).

Carry out a shake-flask fermentation trial with the strains AT-068 and AT-069 with the gene *vhb* and its mutant for expression of vitreoscilla hemoglobin. See Table 20 for the output from shake-flask fermentation by recombinant strains. The results show that: The output may be increased obviously from expression of *vhb* by the recombinant strains both with transformed *vhb*/ pTrc99A and with transformed *vhbM*/ pTrc99A plasmid; moreover, the output is increased more significant by the recombinant strain with with transformed *vhbM*/ pTrc99A plasmid.

Table 20, Output from Shake-flask Fermentation by the Recombinant Strain

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-019 (AT-007-02, Δ_{wecB} promotor::Trc promoter) (reference)	23.0±1.8
AT-068 (AT-019, <i>vhb</i> /pTrc99A)	27.2±1.9
AT-069 (AT-019, <i>vhbM</i> /pTrc99A)	31.5±2.0

Example 9

This implementation example describes *Escherichia coli* strains integrated with pTrc-nanEM and influences of those on the output of N-Acetyl-D-Glucosamine, of which the natural endogenous promoter of the gene *glmS* of Glucosamine-6-Phosphate Synthase (GlmS) and/or the *nagB* gene of D-Glucosamine-6-Phosphate Deaminase (NagB) is replaced and/or deleted, and the gene *vhb* and its mutants for expression of vitreoscilla hemoglobin (Vhb).

In *Escherichia coli* strains integrated with pTrc-nanEM cassette, the natural endogenous promoter of the *nanB* gene is replaced by Trc promoter to produce AT-032 (AT-031-02, Δ *nagB* promoter::Trc promoter), and the natural endogenous promoter of the *glmS* gene is further deleted to produce AT-033 (AT-032, Δ *glmS* promoter); In *Escherichia coli* strains integrated with pTrc-nanEM cassette, the natural endogenous promoter of the *glmS* gene is replaced by Trc promoter to produce AT-034 (AT-031-02, Δ *nagB* promoter::Trc promoter), and the natural endogenous promoter of the *nagB* gene is further deleted to produce AT-035 (AT-034, Δ *nagB* promoter).

Prepare competence of recombinant *Escherichia coli* strain AT-033 and AT-035; then, transform *vhb*/pTrc99A and *vhbM*/pTrc99A plasmids into AT-033 and AT-035 by CaCl₂ transformation method, pick up monoclones and cultivate, and pipet plasmid to identify positive clones.

No. of the obtained strains: AT-070 (AT-033, *vhb*/pTrc99A), AT-071 (AT-033, *vhbM*/pTrc99A), AT-072 (AT-035, *vhb*/pTrc99A), and AT-073 (AT-035, *vhbM*/pTrc99A).

Carry out a shake-flask fermentation trial with the strains AT-070, AT-071, AT-072, and AT-073 with the gene *vhb* and its mutant for expression of vitreoscilla hemoglobin. See Table 21 for the output from shake-flask fermentation by recombinant strains. The results show that: The output may be increased obviously from expression of *vhb* by the recombinant strains both with transformed *vhb*/pTrc99A and with transformed *vhbM*/pTrc99A plasmid; moreover, the output is increased more significant by the recombinant strain with transformed *vhbM*/pTrc99A plasmid.

Table 21, Output from Shake-flask Fermentation by the Recombinant Strain

species	Output of N-Acetyl-D-Glucosamine (g/L)
---------	---

AT-033 (AT-032, Δ glmS promotor) (reference)	12.2 $\pm$ 1.2
AT-070 (AT-033, vhb/pTrc99A)	15.0 $\pm$ 1.2
AT-071 (AT-033, vhbM/pTrc99A)	18.1 $\pm$ 1.3
AT-035 (AT-034, Δ nagB promotor) (reference)	9.8 $\pm$ 0.8
AT-072 (AT-035, vhb/pTrc99A)	13.1 $\pm$ 1.2
AT-073 (AT-035, vhbM/pTrc99A)	16.7 $\pm$ 1.3

Example 10

This implementation example describes Escherichia coli strains integrated with pTrc-nanEM gene cassette, of which the natural endogenous promoter of the wecB gene is replaced by Trc promoter, and the gene vhb thereof and its mutant for expression of vitreoscilla hemoglobin (Vhb) as well as influence on the output of N-Acetyl-D-Glucosamine.

The natural endogenous promoter of the wecB gene of Escherichia coli strains integrated with pTrc-nanKM cassette is replaced by Trc promoter to produce AT-037 (AT-031-02, Δ wecB promotor::Trc promoter).

Prepare competence of recombinant Escherichia coli strain AT-037; then, transform vhb/pTrc99A and vhbM/pTrc99A plasmids into AT-037 by CaCl_2 transformation method, pick up monoclones and cultivate, and pipet plasmid to identify positive clones.

No. of the obtained strains: AT-074 (AT-037, vhb/pTrc99A), and AT-075 (AT-037, vhbM/pTrc99A).

Carry out a shake-flask fermentation trial with the strains AT-074 and AT-075 with the gene vhb and its mutant for expression of vitreoscilla hemoglobin. See Table 22 for the output from shake-flask fermentation by recombinant strains. The results show that: The output may be increased obviously from expression of vhb by the recombinant strains both with transformed vhb/ pTrc99A and with transformed vhbM/ pTrc99A plasmid; moreover, the output is increased more significant by the recombinant strain with with transformed vhbM/ pTrc99A plasmid.

Table 22. Output from Shake-flask Fermentation by the Recombinant Strain

species	Output of
---------	-----------

	N-Acetyl-D-Glucosamine (g/L)
AT-037 (AT-031-02, Δ wecB promoter::Trc promoter) (reference)	13.4 $\pm$ 1.2
AT-074 (AT-037, vhb/pTrc99A)	16.2 $\pm$ 1.2
AT-075 (AT-037, vhbM/pTrc99A)	19.9 $\pm$ 1.3

Example 11

This implementation example describes Escherichia coli strains integrated with pTrc-nanBM and influences of those on the output of N-Acetyl-D-Glucosamine, of which the natural endogenous promoter of the gene glmS of Glucosamine-6-Phosphate Synthase (GlmS) and/or the nagB gene of D-Glucosamine-6-Phosphate Deaminase (NagB) is replaced and/or deleted, and the gene vhb and its mutants for expression of vitreoscilla hemoglobin (Vhb).

In Escherichia coli strains integrated with pTrc-nanBM cassette, the natural endogenous promoter of the nanB gene is replaced by Trc promoter to produce AT-044 (AT-043-02, Δ nagB promoter::Trc promoter), and the natural endogenous promoter of the glmS gene is further deleted to produce AT-045 (AT-044, Δ glmS promoter); In Escherichia coli strains integrated with pTrc-nanBM cassette, the natural endogenous promoter of the glmS gene is replaced by Trc promoter to produce AT-046 (AT-043-02, Δ nagB promoter::Trc promoter), and the natural endogenous promoter of the nagB gene is further deleted to produce AT-047 (AT-046, Δ nagB promoter).

Prepare competence of recombinant Escherichia coli strain AT-045 and AT-047; then, transform vhb/pTrc99A and vhbM/pTrc99A plasmids into AT-045 and AT-047 by CaCl₂ transformation method, pick up monoclones and cultivate, and pipet plasmid to identify positive clones.

No. of the obtained strains: AT-076, AT-077, AT-078 (AT-047, vhb/pTrc99A), and AT-079.

Carry out a shake-flask fermentation trial with the strains AT-076, AT-077, AT-078, and AT-079 with the gene vhb and its mutant for expression of vitreoscilla hemoglobin. See Table 23 for the output from shake-flask fermentation by recombinant strains. The results show that: The output may be increased obviously from expression of vhb by the recombinant strains both with transformed vhb/

pTrc99A and with transformed vhbM/ pTrc99A plasmid; moreover, the output is increased more significant by the recombinant strain with with transformed vhbM/ pTrc99A plasmid.

5 Table 23. Output from Shake-flask Fermentation by the Recombinant Strain

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-045 (AT-044, Δ glmS promotor) (reference)	19.2 $\pm$ 1.2
AT-076 (AT-045, vhb/pTrc99A)	23.0 $\pm$ 1.3
AT-077 (AT-045, vhbM/pTrc99A)	28.1 $\pm$ 1.5
AT-047 (AT-046, Δ nagB promotor) (reference)	15.5 $\pm$ 1.2
AT-078 (AT-047, vhb/pTrc99A)	20.6 $\pm$ 1.3
AT-079 (AT-047, vhbM/pTrc99A)	25.6 $\pm$ 1.4

Example 12

This implementation example describes Escherichia coli strains integrated with pTrc-nanKM gene cassette, of which the natural endogenous promoter of the nanE gene is replaced by Trc promoter, the natural endogenous promoter of the wecB gene and NagB gene is replaced and/or deleted, the natural endogenous promoter of the wecB gene is replaced by Trc promoter, and the gene vhb thereof and its mutant for expression of vitreoscilla hemoglobin (Vhb) as well as influence on the output of N-Acetyl-D-Glucosamine.

15 In Escherichia coli strains integrated with pTrc-nanKM cassette, of which the natural endogenous promoter of the nagB gene is replaced by Trc promoter and meanwhile the natural endogenous promoter of the glmS gene is deleted, the natural endogenous promoter of the nanE gene is replaced by Trc promoter to produce AT-015 (AT-011, Δ nanE promotor::Trc promoter), and the natural endogenous promoter of the wecB gene is further replaced by Trc promoter to produce AT-027 (AT-015, Δ wecB promotor::Trc promoter); In Escherichia coli strains integrated with pTrc-nanKM cassette, of which the natural endogenous promoter of the glmS gene is replaced by Trc promoter and meanwhile the natural endogenous promoter of the

nagB gene is deleted, the natural endogenous promoter of the nanE gene is replaced by Trc promoter to produce AT-017 (AT-013, Δ nanE promoter::Trc promoter), and the natural endogenous promoter of the wecB gene is further replaced by Trc promoter to produce AT-029 (AT-017, Δ wecB promoter::Trc promoter);

Prepare competence of recombinant Escherichia coli strain AT-027 and AT-029; then, transform vhb/pTrc99A and vhbM/pTrc99A plasmids into AT-027 and AT-029 by CaCl₂ transformation method, pick up monoclones and cultivate, and pipet plasmid to identify positive clones.

No. of the obtained strains: AT-080 (AT-027, vhb/pTrc99A), AT-081 (AT-027, vhbM/pTrc99A), AT-082 (AT-029, vhb/pTrc99A), and AT-083 (AT-029, vhbM/pTrc99A).

Carry out a shake-flask fermentation trial with the strains AT-080, AT-081, AT-082, and AT-083 with the gene vhb and its mutant for expression of vitreoscilla hemoglobin. See Table 24 for the output from shake-flask fermentation by recombinant strains. The results show that: The output may be increased obviously from expression of vhb by the recombinant strains both with transformed vhb/pTrc99A and with transformed vhbM/pTrc99A plasmid; moreover, the output is increased more significant by the recombinant strain with with transformed vhbM/pTrc99A plasmid.

Table 24. Output from Shake-flask Fermentation by the Recombinant Strain

species	Output of N-Acetyl-D-Glucosamine (g/L)
AT-027 (AT-015, Δ wecB promoter::Trc promoter) (reference)	39.8±2.0
AT-080 (AT-027, vhb/pTrc99A)	46.5±2.1
AT-081 (AT-027, vhbM/pTrc99A)	49.5±2.0
AT-029 (AT-017, Δ wecB promoter::Trc promoter) (reference)	40.2±2.1
AT-082 (AT-035, vhb/pTrc99A)	47.7±2.2
AT-083 (AT-035, vhbM/pTrc99A)	51.6±2.3

Example 13

This implementation example describes a fermentation trial for production of N-Acetyl-D-Glucosamine by 10-L fermentation tank

Carry a fermentation trial for production of N-Acetyl-D-Glucosamine by 10-L fermentation tank, using recombinant engineering strain AT-083 as production strain.

1. Seed Cultivation

(1) Cultivation of Primary Seed: Pick monoclonal strain freshly cultivated in the LB plate medium, inoculate into 8 mL of LB broth medium, and shake-cultivate at 37°C and 225 rpm for 8 h.

(2) Cultivation of Secondary Seed: Transfer 6 mL of the primary seed culture solution, inoculate into 1000-mL shake flask containing 200 mL of M9 culture solution, and shake-cultivate at 37°C and 225rpm for 16 h, until OD₆₀₀ value is 6.0-10, approximately the medium stage of log growth.

(3) Prepare the fermentation medium according to Table 25, where the microelement solution is prepared according to Table 26, and the complex vitamins solution is prepared according to Table 27.

Table 25. Fermentation Medium

Ingredients	Amount (/L)
K ₂ HPO ₄	1.30g
KH ₂ PO ₄	1.00g
MgSO ₄ ·7H ₂ O	0.10g
NH ₄ Cl	0.02g
(NH ₄) ₂ SO ₄	0.20g
NaH ₂ PO ₄	0.60g
Polyether Defoamer	10 mL
Microelement Solution	4ml
Complex Vitamins Solution	4ml
Glucose	6.00g

NOTE:

①The microelement solution is sterilized separately and then added, and the vitamins solution is filtered and then added;

②Glucose: Concentration 65%(w/v); it is sterilized separately and is added prior to inoculation. Amount to be added: 6.0 g/L;

③The above solutions are combined, and then adjust to pH 7.0 with 10M NH₄OH;

④The fermentation medium is a basal medium prior to addition of glucose; initial loading amount of the basal medium (initial volume accounting for the total capacity of the fermentation tank): 50%.

Table 26. Microelement Solution

Ingredients	Amount used (g/L)
CaCl ₂ · 2H ₂ O	10
FeCl ₃ · 6H ₂ O	10
MnSO ₄ · 5H ₂ O	2.5
AlCl ₃ · 6H ₂ O	2.5
CoCl ₂ · 6H ₂ O	1.75
ZnSO ₄ · 2H ₂ O	0.5
NaMoO ₄ · 2H ₂ O	0.5
CuSO ₄ · 5H ₂ O	0.25
H ₃ BO ₃	0.125
pH	3 - 4

Table 27. Complex Vitamins Solution

Ingredients	Amount (mg/L)
Folic Acid	2
Vitamin B ₂ Riboflavin	100
Vitamin B ₁ Thiamine HCl	1500
Nicotinic Acid	500
Vitamin B ₆ Pyridoxine HCl	500
Calcium Pantothenate, Ca-panthothenate	500
Biotin	1
Vitamin B ₁₂	10

2. Inoculation

Inoculate the secondary seed solution to the fermentation tank in the ratio of 40 mL/L; inoculation size: 2.5-5% (v/v); the initial OD₆₀₀ is 0.3-0.5.

3. process parameters

Carry out a high-density fermentation by 10-L self-control fermentation tank, and collect data by software equipped in the machine to realize online control by computer. The control parameters are: The air flow is 0.5-1 vvm.; dissolved oxygen is $\geq 20\%$, to increase regulation of rotational speed and ventilation; temperature 37°C ; pH 7.0, the automatic flow is maintained stable by addition of saturated ammonia water. Supplement glucose when glucose in the basal medium is consumed up, i.e. dissolved oxygen is risen again. Glucose is supplemented in a speed to control residual glucose concentration is not more than 0.45 g/L. The glucose-supplementing solution contains 65% (w/v) of glucose, is added with 2.5% Sodium Gluconate or 6% Ribose. The fermentation is stopped after 60-72 h. Total loading amount: 75%-80%.

4. Example (10-L fermentation tank)

(1) Strain No.: AT-083. Batch No.: 1019.

(2) Concentration of Seed Solution: OD_{600} 为 2.8.

(3) Base Stock: 4L.

(4) Inoculation Size 200 mL.

(5) Glucose supplementation speed: The residual glucose concentration is controlled as not more than 0.45 g/L.

(6) Glucose-supplementing solution: The solution contains glucose in a concentration of 65% (w/v) and is added with 2.5% sodium gluconate.

(7) Track Indicators: Measure OD_{600} and residual glucose content (residual glucose in the fermentation solution).

(8) Product: N-Acetyl-D-Glucosamine. Potency: 72 h, 156 g/L.

Example 14

This implementation example describes the processing process after separation and purification of N-Acetyl-D-Glucosamine and D-Glucosamine Hydrochloride

1. Refinement of N-Acetyl-D-Glucosamine

(1) Deactivation: The fermentation solution is placed at 80°C for 30 min.

(2) Solid-liquid separation: Centrifugate at 4000-8000 rpm, discard the bacterial residue and proteins, and transfer the fermentation solution. It may also be filtered via

ceramic membrane.

(3) Decoloration: Product:Water:Activated Charcoal = 1:(1.5-3):(0.01-0.1); stir for 0.5-5 h.

(4) Desalt: Desalt by electroosmosis. Initial salt concentration of the fermentation loaded into the concentrated chamber tank: 0.01-0.05 mol/L. Flow rate of the dilute-chamber fermentation solution: 40-80L/h; flow rate of the concentrated-chamber fermentation solution: 40-80L/h; the voltage of single membrane pair is 0.5-1.4 V. It may also be desalted by anion or cation ion exchange resin.

(5) Concentration: The fermentation solution desalted is heated at 50-80°C under vacuum conditions (0.095MPa) for 8-15 h until oversaturation, by approximately 4-6 folds.

(6) Concentration: The concentrated fermentation solution is cooled to 25-35°C in water at 25°C, then cooled for 1-3 h to 0-10°C in water at 0°C.. Add anhydrous alcohol (in an amount of approximately 5-20 times the product weight), and stir at 700-1500 rpm for 15min-1h.

(7) Washing: Add anhydrous alcohol (same amount as that of the product) and stir for 10-100rpm, 0.5-2h.

(8) Drying: 50-100°C, 3-10h. Purity: 99.96%. The total yield is 91.5%.

2. Refinement of D-Glucosamine Hydrochloride

(1) Deactivation: The fermentation solution is placed at 80°C for 30 min.

(2) Solid-liquid separation: Centrifugate at 4000-8000 rpm, discard the bacterial residue and proteins, and transfer the fermentation solution. It may also be filtered via ceramic membrane.

(3) Decoloration: Product:Water:Activated Charcoal = 1:(1.5-3):(0.01-0.1); stir for 0.5-5 h.

(4) Desalt: Desalt by electroosmosis. Initial salt concentration of the fermentation loaded into the concentrated chamber tank: 0.01-0.05 mol/L. Flow rate of the dilute-chamber fermentation solution: 40-80L/h; flow rate of the concentrated-chamber fermentation solution: 40-80L/h; the voltage of single membrane pair is 0.5-1.4 V. It may also be desalted by anion or cation ion exchange resin.

(5) Concentration: The fermentation solution desalted is heated at 50-80°C

under vacuum conditions (0.095MPa) for 8-15 h until oversaturation, by approximately 4-6 folds.

(6) Hydrolysis: Introduce the concentrated fermentation solution to an enamel or glass container, add concentrated hydrochloric acid (37%) to a final concentration of 12%-16%, stir thoroughly, and maintain at 70°C for 90 min. Hydrochloric Acid may be used in a recycling way.

(7) Crystalliation: First cool to 25-35°C in water at 25°C, and then cool to 4°C in water at 0°C for 1-3 h.

(8) Washing: Add anhydrous alcohol (same amount as that of the product) and stir for 10-100rpm, 0.5-2h. Centrifugate at 700-1500 rpm for 15-60 min to obtain Glucosamine Hydrochloride; the conversion rate is 89.5%.

(9) Dissolution: Dissolve the washed product in water in an similar volume to that of the original fermentation solution.

(10) Decoloration: Add activated charcoal (in an amount of 1%). Mix for 30 min. Then centrifugate at 700-1500 rpm for 15-60min. Or filter to obtain a colorless filtrate.

(11) Recrystalliation: Evaporate at 50 °C and 55 cmHg vacuum until oversaturation. Add anhydrous alcohol (in an amount of approximately 5-20 times the product weight), and stir at 700-1500 rpm for 15min-1h.

(12) Washing: Add anhydrous alcohol (same amount as that of the product) and stir for 10-100rpm, 0.5-2h. Then centrifugate at 700-1500 rpm for 15-60min.

(13) Drying: 50-100°C, 3-10h. Purity: 99.92%. The total yield is 84.6%.

Although this Invention is described detailedly by common explanations and specific implementation schemes, it may be revised or improved on top of this Invention; this point is as plain as the nose on your face for a technician in this field. Therefore, such revisions or improvements that are not deviated from the spirits of this Invention still fall in the range under protection required by this Invention.

Claims

1. A method for production of N-Acetyl-D-Glucosamine and/or D-Glucosamine by microbial fermentation, including:

A) Cultivation of microorganism in the fermentation medium, where the said microorganism contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb); and

B) Collection of N-Acetyl-D-Glucosamine produced in the cultivation step A).

2. The method of Claim 1, wherein the microorganism is transformed by nucleic acid molecules with at least one nucleotide sequence encoding vitreoscilla hemoglobin (Vhb);

the nucleotide sequence encoding vitreoscilla hemoglobin (Vhb) contains at least one genetic modification that may increase the activities of vitreoscilla hemoglobin (Vhb).

3. The method of Claim 2, wherein said genetic modification contains one or more substitutions at the following corresponding sites of the amino acid sequence SEQ ID NO:61: Methionine at Site 45 is substituted by leucine, cysteine at Site 86 is substituted by glycine, and tyrosine at Site 95 is substituted by serine.

4. the method of Claim 2, wherein the nucleic acid sequence encoding the said vitreoscilla hemoglobin (Vhb) is SEQ ID NO: 64.

5. The method of Claim 2, wherein at least approximately 30% (more preferably at least approximately 50%, further more preferably at least approximately 70%, further more preferably at least approximately 80%, and further more at least approximately 90%, and most preferably at least approximately 95%) of the amino acid sequence of the said vitreoscilla hemoglobin (Vhb) is the same as that of SEQ ID NO:61, where the said vitreoscilla hemoglobin (Vhb) is active; Further preferably,

the said vitreoscilla hemoglobin (Vhb) has the amino acid sequence of SEQ ID NO:61.

6. The method of Claim 2, wherein the gene copies in the recombinant nucleic acid molecules, encoding vitreoscilla hemoglobin (Vhb), are more than or equal to 1; the recombinant nucleic acid molecules contain an endogenous promoter, or a promoter with a higher expression level than that of the natural endogenous promoter; preferably, the promoter with higher level than the natural endogenous promoter is selected from HCE promoter, gap promoter, trc promoter, and T7 promoter; most preferably, the promoter with higher level than the natural endogenous promoter is trc promoter.

7. The method of any one of Claims 1-6, wherein the said microorganism contains one or more of the following genetic modifications:

(1) Contain at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine Kinase (NanK) in microorganism;

(2) Contain at least one genetic modification that may increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase in microorganism;

(3) Contain at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Deaminase in microorganism, and preferably contain at least one genetic modification that may decrease the effects of Glucosamine- 6-Phosphate Synthase;

(4) Contain at least one genetic modification that may increase the effects of D-Glucosamine-6-Phosphate Synthase in microorganism, and preferably contain at least one genetic modification that may decrease the effects of D-Glucosamine- 6-Phosphate Deaminase;

(5) Contain at least one genetic modification that may increase the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) in microorganism.

8. The method of Claim 7, wherein the genetic modification to increase the effects of N-Acetyl-D-Mannosamine Kinase in microorganism are selected from: a) increased effects of N-Acetyl-D-Mannosamine Kinase in microorganism; and/or b) overexpression of N-Acetyl-D-Mannosamine Kinase in microorganism.

5

9. The method of Claim 8, wherein the microorganism is transformed by molecules of at least one recombinant nucleic acid, containing one nucleotide sequence encoding N-Acetyl-D-Mannosamine Kinase; the amino acid sequence encoding N-Acetyl-D-Mannosamine Kinase is SEQ ID NO: 26.

10

10. The method of Claim 7, wherein the genetic modification to increase the effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase in microorganism are selected from: a) increased effects of N-Acetyl-D-Mannosamine-6-Phosphate Epimerase in microorganism; and/or b) overexpression of N-Acetyl-D-Mannosamine- 6-Phosphate Epimerase in microorganism.

15

11. The method of Claim 10, wherein the microorganism is transformed by molecules of at least one recombinant nucleic acid, containing one nucleotide sequence encoding N-Acetyl-D-Mannosamine-6-Phosphate Epimerase;

20

the amino acid sequence encoding the said N-Acetyl-D- Mannosamine-6-Phosphate Epimerase (NanE) is SEQ ID NO: 56.

12. The method of Claim 7, wherein the genetic modification increasing the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase in microorganism is selected from a) increasing the effects of UDP-N-Acetyl-D-Glucosamine-2-Epimerase in microorganism; and/or b) overexpression of UDP-N-Acetyl-D- Glucosamine- 2-Epimerase in microorganism.

25

13. The method of Claim 12, wherein the microorganism is transformed by

molecules of at least one recombinant nucleic acid, containing one nucleotide sequence encoding UDP-N-Acetyl-D-Glucosamine-2-Epimerase; the amino acid sequence encoding UDP-N-Acetyl-D-Glucosamine-2-Epimerase (WecB) is SEQ ID NO: 58.

5

14. The method of any one of Claims 1-13, where the said microorganism contains one or more of the following genetic modifications:

(1) Contain at least genetic modification that may decrease the effects of Mannose transporter EIIM, P/III^{man}(ManXYZ) in microorganism;

10 (2) Contain at least one genetic modification that may decrease the effects of N-Acetylneuraminate Lyase (NanA) in microorganism;

(3) Contain at least one genetic modification that may decrease the effects of N-Acetyl-D-Glucosamine-6-Phosphate Deacetylase (NagA) in microorganism;

15 (4) Contain at least one genetic modification that may decrease the effects of N-Acetyl-D-Glucosamine Specific Enzyme II^{Nag} (NagE) in microorganism;

(5) Contain at least one genetic modification that may increase the effects of PhosphoGlucosamine Mutase (GlmM) in microorganism;

20 (6) Contain at least one genetic modification that may increase the effects of bifunctional N-acetyl Glucosamine-1-Phosphate Uridyltransferase (GlmU) in microorganism.

15. A microorganism which contains at least one genetic modification that may express vitreoscilla hemoglobin (Vhb).

25 16. The microorganism of Claim 15, wherein the microorganism is transformed by nucleic acid molecules with at least one nucleotide sequence encoding vitreoscilla hemoglobin (Vhb), the nucleic acid sequence encoding the said vitreoscilla hemoglobin (Vhb) is SEQ ID NO: 64.

17. One vitreoscilla hemoglobin (Vhb) with higher activities, which has the amino acid sequence as shown in SEQ ID NO:65.

5 18. A nucleic acid molecule encoding vitreoscilla hemoglobin (Vhb) with higher activities according to Claim 17, where the said nucleic acid molecules have the nucleotide sequence as shown in SEQ ID NO: 64.

10 19. A carrier containing said nucleic acid molecules according to Claim 18.

20. A microorganism with genomes containing said nucleic acid molecule according to Claim 18.