

PATENT SPECIFICATION

NO DRAWINGS

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COMPLETE SPECIFICATION

Improvements in or relating to the Production of Micro-organisms

We, THE BRITISH PETROLEUM COMPANY LIMITED, of Britannic House, Finsbury Circus, London E.C.2, a British joint-stock Corporation, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed to be particularly described in and by the following statement:—

This invention relates to a process for the production of micro-organisms, for example, yeasts. This invention also relates to a process for the removal of straight chain hydrocarbons, wholly or in part, from mixtures of said hydrocarbons with other hydrocarbons.

In a process in which a micro-organism is cultivated in the presence of a hydrocarbon it has now been found that the micro-organism may be contaminated by sorbed hydrocarbons. Sometimes other materials formed in the fermentation process may be present, thus yeasts may be contaminated by lipids.

It has also now been found that under certain conditions these contaminants or part thereof will constitute a feedstock for the micro-organism whereby the quantity of said contaminants may be reduced.

In accordance with the present invention there is provided a process which comprises, in a growth stage, cultivating a micro-organism capable of growth in the presence of a hydrocarbon feedstock consisting wholly or in part of straight chain hydrocarbons; wherein said micro-organism is cultivated in the presence of said feedstock; in the presence of an aqueous nutrient medium; and in the presence of a gas containing free oxygen and subsequently, if necessary, separating the micro-organism from the bulk of the unconsumed hydrocarbon whereby there is obtained a micro-organism free of a substantial amount of hydrocarbon but containing a small

amount of hydrocarbon as contaminant, and thereafter in a purification stage maintaining said micro-organism with an aqueous nutrient medium and a gas containing free oxygen, whereby the hydrocarbon contaminating the micro-organism is reduced in quantity or is eliminated.

Preferably the hydrocarbon feedstock contains C_{10} or higher.

Suitably there may be used a hydrocarbon fraction derived from petroleum.

It is well-known that certain petroleum fractions, particularly gas oils, contain straight chain hydrocarbons, mainly paraffins which are waxes and which have an adverse effect upon the pour point of the fractions; that is to say, when these hydrocarbons are removed, wholly or in part, the pour point of the fraction is lowered. Usually the wax is removed by precipitation by means of solvents, the wax originally present in the fraction being recovered as such, that is, without conversion to more valuable products.

The petroleum fractions boiling below the gas oils, for example, heavy naphthenes and kerosines also contain straight chain hydrocarbons which are potentially valuable for conversion to other products but hitherto, in general, utilisation of these hydrocarbons has been rendered difficult by the necessity of recovering these hydrocarbons from the petroleum fractions, in which they are contained, before they can be converted to other products.

According to a preferred feature of this invention there is provided a process which comprises cultivating a micro-organism in the manner as hereinbefore described in the presence of a petroleum fraction consisting in part of straight chain hydrocarbons and having a mean molecular weight corresponding to at least 10 carbon atoms per molecule, and in

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the presence of an aqueous nutrient medium; and in the presence of a gas containing free oxygen and separating from the mixture, on the one hand, the micro-organism and, on the other hand, a petroleum fraction having a reduced proportion of straight chain hydrocarbons or which is free of said straight chain hydrocarbons.

The process of the invention is of particular value for the treatment of petroleum gas oil fractions which contain straight chain hydrocarbons in the form of waxes, since by the process of the invention, a gas oil of improved pour point is obtained while the waxes are converted to a valuable product.

Usually the straight-chain hydrocarbons will be present in the feedstocks according to the invention as paraffins; however, the straight chain hydrocarbons may be present as olefins; also there may be used a mixture containing straight chain paraffins and olefins.

It is an important feature of this invention that when cultivating yeasts in the presence of the feedstocks hereinbefore described under conditions favouring the growth of the yeasts at the expense of the straight chain hydrocarbons, the other hydrocarbons, for example iso-paraffins, naphthenes and aromatics are not metabolised or, at most, the proportion which is metabolised is very small. Furthermore, unlike conventional chemical processes governed by the law of mass action, the rate of removal of straight chain hydrocarbons is not substantially reduced as the proportion of these hydrocarbons in the overall mixture of hydrocarbons decreases (except, of course, in the very final stages of removal). Thus, when desired, the percentage conversion of straight chain hydrocarbons which is achieved can be maintained at a value approaching 100% without necessitating a very disproportionate expenditure of contact time to achieve small improvements. Furthermore, in the continuous process, this high percentage conversion can be achieved without resorting to the use of a long reaction path.

By the application of this process under conditions which limit the metabolism of the straight chain hydrocarbons it is possible to operate with the removal of only a desired proportion of these hydrocarbons.

Suitable feedstocks to the process of the invention include kerosine, gas oils and lubricating oils; these feedstocks may be unrefined or may have undergone some refinery treatment, but will be required to contain a proportion of straight chain hydrocarbons in order to fulfil the purpose of this invention. Suitably the petroleum fraction will contain 3—45% by weight of straight chain hydrocarbons.

Micro-organisms which are cultivated as herein described may be yeasts, moulds or bacteria.

Preferably when a yeast is employed this is of the family Cryptococcaceae and par-

ticularly of the sub-family Cryptococcoidae; however, if desired there may be used, for example, ascosporogeneous yeasts of the sub-family Saccharomycetoidae. Preferred genera of the Cryptococcoidae family are *Torulopsis* (also known as *Torula*) and *Candida*. Preferred strains of yeast are as follows. In particular it is preferred to use the specific stock of indicated reference numbers; these reference numbers refer to CBS stock held by the Central Bureau voor Schimmelcultuur, Baarn, Holland and to INRA stock held by the Institut National de la Recherche Agronomique, Paris, France.

<i>Candida</i>	<i>lipolytica</i>	
<i>Candida</i>	<i>pulcherrima</i>	CBS 610
<i>Candida</i>	<i>utilis</i>	
<i>Candida</i>	<i>utilis</i> , <i>Variati major</i>	CBS 841
<i>Candida</i>	<i>tropicalis</i>	CBS 2317
<i>Torulopsis</i>	<i>colliscoloja</i>	CBS 133
<i>Hansenula</i>	<i>anomala</i>	CBS 110
<i>Oidium</i>	<i>lactis</i>	
<i>Neurospora</i>	<i>sitophila</i>	
<i>Mycoderma</i>	<i>cancollote</i>	INRA 157V11

Of the above *Candida lipolytica* is particularly preferred.

Suitable moulds are of the family Aspergillaceae. A suitable genus is *Penicillium*.

Preferably there is used *Penicillium expansum*. Another suitable genus is *Aspergillus*.

Penicillium expansum is suitable for cultivation in an aqueous nutrient medium containing hydrocarbons.

Penicillium roqueforti, *Penicillium notatum*, *Aspergillus fustigatus*, *Aspergillus niger* and *Aspergillus versicolor* may be used for cultivation on solid hydrocarbons as feedstock.

Suitably the bacteria are of one of the orders:—

Pseudomonadales, Eubacteriales and Actinomycetales.

Preferably the bacteria which are employed are of the family Bacillaceae and Pseudomonadaceae. Preferred species are *Bacillus megatherium*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. Other strains which may be employed include:—

<i>Bacillus amylolactis</i>	
<i>Pseudomonas natriegens</i>	115
<i>Arthrobacter</i> sp.	
<i>Micrococcus</i> sp.	
<i>Corynebacterium michiganense</i>	
<i>Pseudomonas syringae</i>	
<i>Xanthomonas begoniae</i>	120
<i>Flavobacterium</i> sp. <i>devorans</i>	
<i>Acetobacter</i> sp.	
<i>Actinomyces</i> sp.	
<i>Agrobacterium</i> sp.	
<i>Aplanobacter</i> sp.	125

For the growth of the micro-organism it will be necessary to provide, in addition to the feedstock, an aqueous nutrient medium and a supply of oxygen, preferably in the form of air.

Cryptococcoidae
Saccharomycetoidae

Cryptococcoidae
colliscoloja

Aspergillaceae

Bacillaceae
Pseudomonadaceae
Bacillus subtilis

amylolactis

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A typical nutrient medium for the growth of *Nocardia* has the following composition:—

		grams
5	Ammonium sulphate - - -	1
	Magnesium sulphate - - -	0.20
	Ferrous sulphate, $7H_2O$ - - -	0.005
	Manganese sulphate, $1H_2O$ - - -	0.002
	Monopotassium phosphate - - -	2
	Disodium phosphate - - -	3
10	Calcium chloride - - -	0.1
	Sodium carbonate - - -	0.1
	Yeast extract - - -	0.008
	Distilled water (to make up to 1000 mls).	

For other bacteria a suitable nutrient medium has the composition.

15	Monopotassium phosphate - - -	7 gm
	Magnesium sulphate, $7H_2O$ - - -	0.2 gm
	Sodium chloride - - -	0.1 gm
	Ammonium chloride - - -	2.5 gm
20	Tap water (trace elements) - - -	100 mls
	Yeast extract - - -	0.025 gm
	made up to 1000 mls with distilled water	

A suitable nutrient medium for yeasts (and moulds) has the composition:—

25		grams
	Diamonium phosphate - - -	2
	Potassium chloride - - -	1.15
	Magnesium sulphate, $7H_2O$ - - -	0.65
	Zinc sulphate - - -	0.17
30	Manganese sulphate, $1H_2O$ - - -	0.045
	Ferrous sulphate, $7H_2O$ - - -	0.068
	Tap water - - -	200
	Yeast extract - - -	0.025
	Distilled water (to make up to 1000 mls)	

35 Micro-organisms, and in particular yeasts, when first cultivated with the use of hydrocarbon fractions as feedstock sometimes grow with difficulty and it is sometimes necessary to use an inoculum of a micro-organism which has previously been adapted for growth on the hydrocarbon fraction which it is intended to use. Furthermore the micro-organism although cultivated in the presence of an aqueous mineral medium containing the appropriate nutrient elements may grow with difficulty, because the hydrocarbon fraction does not contain the growth factors which exist in carbohydrate feedstocks, unless these growth factors are added.

50 The growth of the micro-organism used is favoured by the addition to the culture medium of a very small proportion of extract of yeast (an industrial product rich in vitamins of group B obtained by the hydrolysis of a yeast) or more generally of vitamins of group B and/or biotin. This quantity is preferably of the order of 25 parts per million with reference to the aqueous fermentation medium. It can be higher or lower according to the conditions chosen for the growth.

60 The growth of the micro-organism takes place at the expense of the feedstock fraction with the intermediate production of bodies having an acid function, principally fatty acids, in such manner that the pH of the

aqueous mineral medium progressively diminishes. If one does not correct it the growth is fairly rapidly arrested and the concentration of the micro-organism in the medium, that is cellular density, no longer increases so that there is reached a so-called stationary phase.

Preferably therefore the aqueous nutrient medium is maintained at a desired pH by the step-wise or continuous addition of an aqueous medium of high pH value. Usually, when using moulds or yeasts and in particular when using *Candida lipolytica*, the pH of the nutrient medium will be maintained in the range 3—6 and preferably in the range 4—5. (Bacteria require a higher pH, usually 6.5—8). Suitable alkaline materials for addition to the growth mixture include sodium hydroxide, potassium hydroxide, disodium hydrogen phosphate and ammonia, either free or in aqueous solution.

The optimum temperature of the growth mixture will vary according to the type of micro-organism employed and will usually lie in the range 25—35°C. When using *Candida lipolytica* and preferred temperature range is 28—32°C.

The take-up of oxygen is essential for the growth of the micro-organism. The oxygen will usually be provided as air. In order to maintain a rapid rate of growth the air, used to provide oxygen, should be present in the form of fine bubbles under the action of stirring. The air may be introduced through a sintered surface. However there may be used the system of intimate aeration known as "vortex aeration".

It has been found that by the use of yeast of the strain *Candida lipolytica* in a process according to the invention in which aeration is effected by "vortex aeration", a high growth rate is achieved whereby the generation time lies in the range 2—5 hours and the cell concentration is increased by a factor of up to 12 in two days.

In batch operation, the micro-organism will usually grow initially at a low rate of increase in cellular density. (This period of growth is referred to as the "lag phase"). Subsequently the rate of growth will increase to a higher rate of growth; the period at the higher rate of growth is referred to as the "exponential phase" and subsequently again the cellular density will become constant (the "stationary phase").

A supply of the micro-organism for starting the next batch will preferably be removed before the termination of the exponential phase.

The growth operation will usually be discontinued before the stationary phase.

At this stage the micro-organism will usually be separated from the bulk of the unused feedstock fraction.

According to one method of treating the

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product the major part of the continuous aqueous phase is first separated; preferably this is carried out by centrifuging, or decanting. The separated aqueous phase will usually contain a greater concentration of non-nutritive ions than can be tolerated in the recycle stream and when this is so, only a proportion of the recovered aqueous phase can be recycled. Thus it will usually be possible to separate ca. 96% by wt. of the aqueous phase which is present in the product, of which on the same percentage basis, ca. 20% by wt. will be discarded. The recycle stream is supplied with make-up quantities of the necessary nutrients and is returned to the fermenter; if desired the make-up materials may be fed to the fermenter as a separate stream.

By centrifuging the product from the growth stage (preferably after decanting as described) three fractions are recovered. These are in order of increasing density:—

- (i) an oil phase containing micro-organism cells
- (ii) an aqueous phase containing traces of oil and micro-organism, and
- (iii) a micro-organism "cream" consisting of micro-organism, having a quantity of oil fixed on to the cells, together with aqueous phase.

Fraction (iii) will then be mixed with an aqueous nutrient medium, which may be the same as or different from the aqueous nutrient medium employed in the growth stage, and is maintained in admixture with a gas containing free oxygen whereby the hydrocarbon contaminating the micro-organism is reduced in quantity or is eliminated.

Again, preferably, the major part of the continuous aqueous phase is separated with recycle of a proportion of the recovered aqueous phase, as hereinbefore described; recycle may be to either the growth stage or purification stage as desired.

By centrifuging the product from the purification stage, preferably after separating the major part of the aqueous phase by decanting, there are obtained

- (i) an aqueous phase containing traces of micro-organism
- (ii) a micro-organism cream.

If necessary this cream may be further treated for the removal of traces of oil which may be held to the cells. To this end, the micro-organism cream may be washed with an aqueous solution of a surfactant and thereafter with water.

Finally the yeast may be dried under conditions suitable for its subsequent use as a foodstuff.

When operating continuously it will usually be necessary to provide separate fermenters or separate zones in the same fermenter for the operation of the growth and purification stages.

Other steps which may be taken to obtain

a purified micro-organism or a product derived therefrom or to improve the process in respect of the production of the unmetabolised hydrocarbon fraction are described in the following applications; the use of any process conditions therein described lies within the scope of the present invention.

Preferred methods for use in the cultivation of the micro-organism and for the recovery of the product are described in British Patent Specification No. 914567, 1021697 and 1021698—also in British Patent Application Nos:—

	Serial Nos.
49049/62	1,059,881
49050/62	1,059,882
49052/62	1,059,883
49055/62	1,059,884
49056/62	1,059,885
49060/62	1,059,887
49061/62	1,059,888
49063/62	1,059,889
2234/63	1,059,890
25210/63	1,049,065.

The stages of the process may be carried out entirely batchwise. However, if desired, any one or more stages herein described may be carried out in continuous manner.

The invention is illustrated but not limited with reference to the following examples.

Throughout these examples cellular density is expressed as dry weight of yeast per litre of culture.

EXAMPLE 1

40 litres of an aqueous mineral nutrient medium having the composition given below were introduced in a stainless steel fermenter having an effective capacity of 60 litres.

In order to keep the temperature in the fermenter constant at 30°C, water was circulated in an annulus constituted by the space between two concentric cylinders, the smaller one being the fermenter itself.

The aqueous nutrient medium had the composition:—

	grams
Diammonium phosphate	2
Potassium chloride	1.15
Magnesium sulphate, 7H ₂ O	0.65
Zinc sulphate	0.17
Manganese sulphate, 1H ₂ O	0.045
Ferrous sulphate, 7H ₂ O	0.068
Yeast extract	0.025
Tap water	200
Distilled water add.	1000 ml.

20 litres of a 24 hour inoculum of *Candida lipolytica* on mixed C₆—C₁₀ normal hydrocarbons were then added, such that the cellular density was about 1 gm of dry matter per litre.

1.03 litres of heavy gas-oil, that is 15



grams/litre, were then added, this quantity being sufficient to take the cellular density to 2 grams/litre.

The temperature of the culture was kept at $30 \pm 1^\circ\text{C}$, pH 4 and aeration and agitation giving 3 millimoles of O_2 per litre of medium per minute. 10N ammonia was admitted by an automatic pH controller.

When the flow of ammonia reached 20 ml. the addition of gas-oil was begun assuming dry yeast produced a yield on gas-oil of $(100 \times \frac{\text{gas-oil feed}}{\text{dry yeast produced}})$

of 10% by weight and a cell division time of 3 hours. This addition was carried out every hour until 200 grams/litre, i.e. 13.8 litres had been added.

Starting with a cellular density of 2 grams/litre at 25 hours at the end of the exponential growth phase, a density of 15 grams/litre was obtained. This value remained constant during the stationary phase from 25 to 40 hours. During this period the yeasts were lacking in carbon feedstock, other elements being present in excess. It acted as a phase of starvation.

The yeasts were recovered at 25 hours and at 40 hours by centrifuging and washing at $+5^\circ\text{C}$. They were then dried by lyophilisation and analysed. Their total lipid content (fraction soluble in hexane) was 18% when recovered after 25 hours and 3% when recovered after 40 hours.

EXAMPLE 2

A similar experiment was carried out to that described in Example 1 except that the yeast was recovered from the culture liquor at 25 hours and then placed in 60 litres of fresh mineral medium, without hydrocarbon, in the same fermenter. The mixture was then stirred for 10 hours under the same culture conditions (temperature 30°C , pH at 4 and aeration at 3 millimoles O_2 per litre per hour). The cellular density was of the order of 15 grams/litre at the start, and remained constant throughout the process. Even though the yeast contained 20% of total lipids (based on dry weight of yeast) at the moment of introduction into the new medium, after the starvation treatment it did not contain more than 8% by weight.

EXAMPLE 3

A similar experiment to that described in Examples 1 and 2 was carried out on yeasts from continuous fermentation, but in this case it was necessary to work with washed yeasts in a fresh medium, as in the continuous process one cannot obtain a racking-off liquor entirely free of assimilable carbon substrate.

The culture in the 60 litre fermenter was stabilised at 0.1 litres/hour of total mineral

medium plus gas-oil per litre of culture in the fermenter.

The operating conditions having been fixed (in particular temperature at 30°C and pH at 4), the yeast was removed from part of the culture by centrifugation at $+5^\circ\text{C}$. After washing it was put into a 20 litre fermenter containing 15 litres of the mineral medium given in Example 1, to give a cellular density of 20 grams/litre.

The pH was regulated automatically to 4, the temperature to 30°C and the air flow and agitation so as to give 3 millimoles of oxygen per litre of culture per minute. The treatment was applied for 10 hours. The cellular density remained at 20 grams/litre throughout the experiment. The yeasts were analysed for their fat content at the beginning and end of the process. A marked fall in fat content was observed, from 15 to 5% by wt. on dry matter.

WHAT WE CLAIM IS:—

1. A process which comprises, in a growth stage, cultivating a micro-organism capable of growth in the presence of a hydrocarbon feedstock consisting wholly or in part of straight chain hydrocarbons, wherein said micro-organism is cultivated in the presence of said feedstock, in the presence of an aqueous nutrient medium; and in the presence of a gas containing free oxygen and subsequently, if necessary, separating the micro-organism from the bulk of the unconsumed hydrocarbon whereby there is obtained a micro-organism free of a substantial amount of hydrocarbon but containing a small amount of hydrocarbon as contaminant, and thereafter in a purification stage maintaining said micro-organism with an aqueous nutrient medium and a gas containing free oxygen, whereby the hydrocarbon contaminating the micro-organism is reduced in quantity or is eliminated.

2. A process according to claim 1 in which the growth stage and the purification stage are carried out continuously and in which the growth stage and the purification stage are carried out in separate fermenters or in different zones of the same fermenter.

3. A process according to either of the preceding claims in which the micro-organism which is cultivated is a yeast.

4. A process according to claim 3 in which the yeast is of the family Cryptococcaceae.

5. A process according to claim 4 in which the yeast is of the sub-family Cryptococcoideae.

6. A process according to claim 5 in which the yeast is of the genus *Torulopsis*.

7. A process according to claim 4 in which the yeast is of the genus *Candida*.

8. A process according to claim 7 in



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grams/litre, were then added, this quantity being sufficient to take the cellular density to 2 grams/litre.

The temperature of the culture was kept at $30 \pm 1^\circ\text{C}$, pH 4 and aeration and agitation giving 3 millimoles of O_2 per litre of medium per minute. 10N ammonia was admitted by an automatic pH controller.

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a yield on gas-oil of $(100 \times \frac{\text{gas-oil feed}}{\text{gas-oil feed}})$

of 10% by weight and a cell division time of 3 hours. This addition was carried out every hour until 200 grams/litre, i.e. 13.8 litres had been added.

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WHAT WE CLAIM IS:—

1. A process which comprises, in a growth stage, cultivating a micro-organism capable of growth in the presence of a hydrocarbon feedstock consisting wholly or in part of straight chain hydrocarbons, wherein said micro-organism is cultivated in the presence of said feedstock, in the presence of an aqueous nutrient medium; and in the presence of a gas containing free oxygen and subsequently, if necessary, separating the micro-organism from the bulk of the unconsumed hydrocarbon whereby there is obtained a micro-organism free of a substantial amount of hydrocarbon but containing a small amount of hydrocarbon as contaminant, and thereafter in a purification stage maintaining said micro-organism with an aqueous nutrient medium and a gas containing free oxygen, whereby the hydrocarbon contaminating the micro-organism is reduced in quantity or is eliminated.

2. A process according to claim 1 in which the growth stage and the purification stage are carried out continuously and in which the growth stage and the purification stage are carried out in separate fermenters or in different zones of the same fermenter.

3. A process according to either of the preceding claims in which the micro-organism which is cultivated is a yeast.

4. A process according to claim 3 in which the yeast is of the family Cryptococcaceae.

5. A process according to claim 4 in which the yeast is of the sub-family Cryptococcoideae.

6. A process according to claim 5 in which the yeast is of the genus Torulopsis.

7. A process according to claim 4 in which the yeast is of the genus Candida.

8. A process according to claim 7 in

which the yeast is *Candida lipolytica*.

9. A process according to either of claims 1 or 2 in which the micro-organism is a bacterium.

5 10. A process according to any one of the preceding claims in which the feedstock contains at least one straight chain hydrocarbon having at least 10 carbon atoms per molecule.

10 11. A process according to claim 10 in which the feedstock is a petroleum fraction.

12. A process according to any one of the preceding claims in which straight chain hydrocarbons are removed from a petroleum fraction with production of a micro-organism, wherein the feedstock employed for the growth of the micro-organism is a petroleum fraction consisting in part of straight chain hydrocarbons, wherein there is recovered from the product of the growth of the micro-organism
20 a petroleum fraction having a reduced propor-

tion of straight chain hydrocarbons or which is free of said straight chain hydrocarbons.

13. A process according to claim 12 for the removal, wholly or in part, of waxes from
25 a wax-containing petroleum gas oil, wherein the feedstock is a wax-containing petroleum gas oil and wherein there is recovered, from the product of the growth of the micro-organism, a gas oil of reduced content of
30 wax.

14. A process substantially as hereinbefore described with reference to any one of the foregoing Examples.

15. A micro-organism whenever produced
35 by a process as claimed in any one of the preceding claims.

J. WOOLARD,
Agent for the Applicants,
Chartered Patent Agent. *Dg*

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