Hence, there is a need for efficient replication of vectors comprising homopolymeric sequences with high fidelity.

Summary

A solution to these problems is the provision of covalently closed circular recombinant DNA molecules in which the homopolymeric sequence is not located directly next to the origin of replication in the direction of replication, but in which there is a certain distance between the homopolymeric sequence and the origin or replication.

~~In one aspect, the present disclosure refers to covalently closed circular recombinant DNA molecule comprising: a bacterial origin of replication, an insert comprising a homopolymeric region, wherein the homopolymeric region comprises at least one homopolymeric sequence comprising a stretch of at least 15 identical nucleotides and is located at a distance of at least 500 bp from the bacterial origin of replication in the direction of replication wherein the homopolymeric region comprises at least one poly(A) sequence and wherein the poly(A) sequence comprises a sequence of about 20 to about 400 adenosine nucleotides.~~

Accordingly, disclosed herein is a covalently closed circular recombinant DNA molecule comprising:
- an origin of replication,
- an insert comprising a homopolymeric region,
wherein the homopolymeric region is located at a distance of least 500 bp from the origin of replication in the direction of replication.

Also disclosed herein is a covalently closed circular recombinant DNA molecule comprising:
- an origin of replication,
- an insert comprising a homopolymeric region,
wherein the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the origin of replication.

The covalently closed circular recombinant DNA molecule in which the homopolymeric region is located at a distance of least 500 bp from the origin of replication in the direction of replication may improve the yield of the covalently closed circular recombinant DNA molecule in fermentative production compared to the yield of a covalently closed circular

same as the direction of replication of the origin of replication is increased compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the origin of replication. Moreover, the fermentation time is shortened, compared to the fermentation time of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the origin of replication.

Further disclosed herein is a method that the sequence of the covalently closed circular recombinant DNA molecule remains stable during fermentative production.
For example, the homopolymeric region remains stable during fermentative production.
In ~~another~~ one aspect, the present disclosure refers to the use of a covalently closed circular recombinant DNA molecule for increasing the yield of the covalently closed circular recombinant DNA molecule in fermentative production compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is located at a distance of less than 500 bp from the bacterial origin of replication in the direction of replication and the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication, wherein the covalently closed circular recombinant DNA molecule comprising: a bacterial origin of replication, an insert comprising a homopolymeric region, wherein the homopolymeric region comprises at least one homopolymeric sequence comprising a stretch of at least 15 identical nucleotides and is located at a distance of at least 500 bp from the bacterial origin of replication in the direction of replication and wherein the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the bacterial origin of replication. ~~In yet another aspect, the present disclosure refers to the use of a covalently closed circular recombinant DNA molecule as disclosed herein for increasing the yield of the covalently closed circular recombinant DNA in fermentative production compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is located at a distance of less than 500 bp from the bacterial origin of replication in the direction of replication. In a further example, the present disclosure refers to the use of a covalently closed circular recombinant DNA molecule disclosed herein for increasing the yield of the covalently closed circular recombinant DNA in fermentative production compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication.~~

In a further aspect, there is disclosed a method for fermentative production of a covalently closed recombinant DNA molecule comprising the steps of (a) providing a microorganism comprising the covalently closed circular recombinant DNA molecule, wherein the microorganism is a bacterium, wherein the covalently closed circular recombinant DNA molecule comprising: a bacterial origin of replication, an insert comprising a homopolymeric region, wherein the homopolymeric region comprises at least one homopolymeric sequence comprising a stretch of at least 15 identical nucleotides and is located at a distance of at least 500 bp from the bacterial origin of replication in the direction of replication, wherein the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the bacterial origin of replication, and; (b) fermenting the microorganism of step (a).

In yet another aspect, the present disclosure refers to a method for improving the yield of a covalently closed circular recombinant DNA molecule comprising the following steps: (a) providing a covalently closed circular recombinant DNA molecule having a bacterial origin

of replication, and an insert comprising a homopolymeric region comprising at least one homopolymeric sequence comprising a stretch of at least 15 identical nucleotides, wherein the homopolymeric region is located at a distance of less than 500 bp from the bacterial origin of replication in the direction of replication and the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication; (b) modifying the covalently closed circular recombinant DNA molecule of (a) so that the insert comprising a homopolymeric region sequence is located at a distance of at least 500 bp from the bacterial origin of replication in the direction of replication and wherein the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the bacterial origin of replication.

~~In yet another aspect, the present disclosure refers to a method for improving the yield of a covalently closed circular recombinant DNA molecule comprising the following steps: (a) providing a covalently closed circular recombinant DNA molecule having a bacterial origin of replication, and an insert comprising a homopolymeric region comprising at least one homopolymeric sequence comprising a stretch of at least 15 identical nucleotides, wherein the insert comprising the homopolymeric region sequence is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication, and wherein the homopolymeric region is located at a distance of at least 500 bp from the bacterial origin of replication in the direction of replication; (b) modifying the covalently closed circular recombinant DNA molecule of (a) so that the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the bacterial origin of replication.~~

In a further aspect, the present disclosure refers to ~~the use of the covalently closed circular recombinant DNA molecule disclosed herein for the *in vitro* transcription of RNA~~an *in vitro* transcription method of producing RNA comprising the steps of: (a) obtaining RNA by DNA-dependent *in vitro* transcription of a linearized plasmid DNA template using a DNA-dependent RNA polymerase, wherein the plasmid DNA comprises a promoter for a DNA-dependent RNA polymerase, an insert comprising a homopolymeric region, wherein the homopolymeric region comprising a stretch of at least 15 identical nucleotides, wherein the homopolymeric region is located at a distance of at least 500 bp from the origin of replication in the direction of replication and wherein the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the origin of replication; and (b) purifying the RNA.

Disclosed herein is a method for improving the yield of a covalently closed circular recombinant DNA molecule comprising the following steps:

(a) providing a covalently closed circular recombinant DNA molecule having
- an origin of replication, and
- an insert comprising a homopolymeric region, wherein the homopolymeric region is located at a distance of less than 500 bp from the origin of replication in the direction of replication;

(b) modifying the covalently closed circular recombinant DNA molecule of (a) so that the homopolymeric region is located at a distance of at least 500 bp from the origin of replication in the direction of replication.

CLAIMS

~~1. Covalently closed circular recombinant DNA molecule comprising:~~
~~a bacterial origin of replication,~~
~~an insert comprising a homopolymeric region,~~
~~wherein the homopolymeric region comprises at least one homopolymeric sequence comprising a stretch of at least 15 identical nucleotides and is located at a distance of at least 500 bp from the bacterial origin of replication in the direction of replication,~~
~~wherein the homopolymeric region comprises at least one poly(A) sequence and wherein the poly(A) sequence comprises a sequence of about 20 to about 400 adenosine nucleotides.~~

~~2. Covalently closed circular recombinant DNA molecule of claim 1, wherein the homopolymeric region comprises at least one poly(C) sequence and the at least one poly(A) sequence.~~

~~3. Covalently closed circular recombinant DNA molecule of claim 1 or 2, wherein the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the bacterial origin of replication.~~

~~4. Covalently closed circular recombinant DNA molecule according to any one of the previous claims, wherein the yield of the covalently closed circular recombinant DNA molecule in fermentative production is increased compared to the yield of a covalently closed circular recombinant DNA molecule in which the homopolymeric region is located at a distance of less than 500 bp from the bacterial origin of replication in the direction of replication.~~

~~5. Covalently closed circular recombinant DNA molecule according to claim 3, wherein the yield of the covalently closed circular recombinant DNA molecule in fermentative production is increased compared to the yield of a covalently closed~~

~~circular recombinant DNA molecule in which the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication.~~

~~6. Covalently closed circular recombinant DNA molecule according to any one of the previous claims, wherein the covalently closed circular recombinant DNA molecule is selected from the group consisting of plasmid, cosmid, bacterial artificial chromosome (BAC), bacteriophage, viral vector or hybrids thereof.~~

~~7. Covalently closed circular recombinant DNA molecule according to any one of the previous claims, wherein the covalently closed circular recombinant DNA molecule is a plasmid.~~

~~8. Covalently closed circular recombinant DNA molecule according to any one of the previous claims, wherein the bacterial origin of replication is a high copy number origin.~~

~~9. Covalently closed circular recombinant DNA molecule according to any one of the previous claims, wherein the bacterial origin of replication is derived from the pBR322 plasmid, pUC plasmid, pMB1 plasmid, ColE1 plasmid, R6K plasmid, p15A plasmid, or pSC101 plasmid.~~

~~10. Covalently closed circular recombinant DNA molecule according to any one of the previous claims, wherein the bacterial origin of replication is derived from the pUC plasmid.~~

~~11. Covalently closed circular recombinant DNA molecule according to any one of the previous claims, wherein the covalently closed circular recombinant DNA molecule further comprises a primosome assembly site in the heavy strand (PAS-BH).~~

~~12. Covalently closed circular recombinant DNA molecule according to any one of the previous claims, wherein the covalently closed circular recombinant DNA molecule further comprises a selection marker.~~

~~13. Covalently closed circular recombinant DNA molecule according to claim 11, wherein the selection marker is an antibiotic resistance gene.~~

~~14. Covalently closed circular recombinant DNA molecule according to claim 11, wherein the selection marker is a sucrose selectable marker.~~

~~15. Covalently closed circular recombinant DNA molecule according to claim 7, wherein the plasmid is derived from the backbone having the sequence identified in SEQ ID NO: 4 or having the sequence identified in SEQ ID NO: 5.~~

~~16. Covalently closed circular recombinant DNA molecule according to claim 1, wherein the poly(A) sequence comprises a sequence of about 60 to about 250 adenosine nucleotides.~~

~~17. Covalently closed circular recombinant DNA molecule according to claim 2, wherein the poly(C) sequence comprises a sequence of about 15 to 200 cytidine nucleotides.~~

~~18. Covalently closed circular recombinant DNA molecule according to claim 17, wherein the poly(C) sequence comprises a sequence of about 20 to 40 cytidine nucleotides.~~

1.~~19.~~ Use of a covalently closed circular recombinant DNA molecule for increasing the yield of the covalently closed circular recombinant DNA molecule in fermentative production compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric

region is located at a distance of less than 500 bp from the bacterial origin of replication in the direction of replication and the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication, wherein the covalently closed circular recombinant DNA molecule comprising:
- a bacterial origin of replication,
- an insert comprising a homopolymeric region,
wherein the homopolymeric region comprises at least one homopolymeric sequence comprising a stretch of at least 15 identical nucleotides and is located at a distance of at least 500 bp from the bacterial origin of replication in the direction of replication and wherein the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the bacterial origin of replication.~~.~~

~~20. Use of a covalently closed circular recombinant DNA molecule as defined in any one of claims 1 to 18 for increasing the yield of the covalently closed circular recombinant DNA molecule in fermentative production compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is located at a distance of less than 500 bp from the bacterial origin of replication in the direction of replication.~~

2.~~21.~~ Method for fermentative production of a covalently closed recombinant DNA molecule comprising the steps of:
(a) providing a microorganism comprising the covalently closed circular recombinant DNA molecule, wherein the microorganism is a bacterium, wherein the covalently closed circular recombinant DNA molecule comprising:
- a bacterial origin of replication,
- an insert comprising a homopolymeric region,

wherein the homopolymeric region comprises at least one homopolymeric sequence comprising a stretch of at least 15 identical nucleotides and is located at a distance of at least 500 bp from the bacterial origin of replication in the direction of replication, and wherein the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the bacterial origin of replication;

(b) fermenting the microorganism of step (a) in a fed-batch method.

3.~~22.~~ The method according to claim 2~~1~~, further comprising a step of

(c) isolating the covalently closed circular recombinant DNA molecule from the microorganism of step(b).

4.~~23.~~ Method of claim 2 or 3~~21 or 22~~, wherein the microorganism is a bacterium containing a covalently closed circular recombinant temperature inducible high copy DNA plasmid and step (b) comprises the following steps:

(i) growing the bacteria containing a covalently closed circular recombinant temperature inducible high copy DNA plasmid at a temperature in the range of 25°C to 32°C during the growth phase of the fed-batch phase wherein the substrate is supplied at a rate such that the growth rate is μ=0.05 to 0.3hr^{-1} during the fed-batch phase,

(ii) inducing production of said DNA plasmid after the growth phase by increasing the temperature to 36°C to 45°C; and

(iii) continuing fermentation at the temperature applied (ii) to accumulate the said DNA plasmid.

5.~~24.~~ Method according to claim 4~~23~~, wherein the temperature applied in step (i) is about 30°C and the temperature applied in step (ii) is about 42°C.

~~25. Method according to any one of claims 21 to 24, wherein the yield of the covalently closed circular recombinant DNA molecule according to claims 1 to 18 is~~

~~increased compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising homopolymeric region is located at a distance of less than 500 bp from the bacterial origin of replication in the direction of replication.~~

~~26. Method according to any one of claims 21 to 24, wherein the yield of the covalently closed circular recombinant DNA molecule as defined in any one of claims 1 to 18 is increased compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication.~~

~~27. Method according to claim 25, wherein the fermentation time is shortened, compared to the fermentation time of a covalently closed circular recombinant DNA molecule in which the insert comprising homopolymeric region is located at a distance of less than 500 bp from the bacterial origin of replication in the direction of replication.~~

~~28. Method according to claim 26, wherein the fermentation time is shortened compared to the fermentation time of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication.~~

6.~~29.~~ Method according to any one of claims 2 to 5~~21 to 28~~, wherein the homopolymeric region sequence remains stable during the fermentation production.

7.~~30.~~ Method according to any one of claims 2 to 6~~21 to 29~~, wherein the sequence of the covalently closed circular recombinant DNA molecule remains stable during fermentation production.

8.~~31.~~ Method for improving the yield of a covalently closed circular recombinant DNA molecule comprising the following steps:

(a) providing a covalently closed circular recombinant DNA molecule having

- a bacterial origin of replication, and

- an insert comprising a homopolymeric region comprising at least one homopolymeric sequence comprising a stretch of at least 15 identical nucleotides, wherein the homopolymeric region is located at a distance of less than 500 bp from the bacterial origin of replication in the direction of replication and the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication;

(b) modifying the covalently closed circular recombinant DNA molecule of (a) so that the insert comprising a homopolymeric region sequence is located at a distance of at least 500 bp from the bacterial origin of replication in the direction of replication and wherein the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the bacterial origin of replication.

~~32. Method for improving the yield of a covalently closed circular recombinant DNA molecule comprising the following steps:~~

~~(a) providing a covalently closed circular recombinant DNA molecule having~~

~~- a bacterial origin of replication, and~~

~~- an insert comprising a homopolymeric region comprising at least one homopolymeric sequence comprising a stretch of at least 15 identical nucleotides, wherein the insert comprising the homopolymeric region sequence is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication, and wherein the homopolymeric region is located at a distance of at least 500 bp from the bacterial origin of replication in the direction of replication;~~

~~(b) modifying the covalently closed circular recombinant DNA molecule of (a) so that the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the bacterial origin of replication.~~

9.~~33.~~ An *in vitro* transcription method of producing RNA comprising the steps of:

(a) obtaining RNA by DNA-dependent *in vitro* transcription of a linearized plasmid DNA template using a DNA-dependent RNA polymerase, wherein the plasmid DNA comprises

- a promoter for a DNA-dependent RNA polymerase,
- an insert comprising a homopolymeric region, wherein the homopolymeric region comprising a stretch of at least 15 identical nucleotides, wherein the homopolymeric region is located at a distance of at least 500 bp from the origin of replication in the direction of replication and wherein the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the origin of replication; and

(b) purifying the RNA.

~~Use of the covalently closed circular recombinant DNA molecule as defined in any one of claims 1 to 18 for the *in vitro* transcription of RNA.~~