J. Microbiol. Biotechnol. (2018), 28(12), 2009–2018
https://doi.org/10.4014/jmb.1805.05066

jmb

# Effect of the *pat*, *fk*, *stpk* Gene Knock-out and *mdh* Gene Knock-in on Mannitol Production in *Leuconostoc mesenteroides*

**Peng Yu-Wei and Jin Hong-Xing***

*School of Chemical Engineering and Technology, Hebei University of Technology, Tianjin 300130, P.R. China*

*Received: May 28, 2018*
*Revised: September 17, 2018*
*Accepted: October 5, 2018*

First published online
October 10, 2018

**Corresponding author*
Phone: +86-22-60200444;
E-mail: jinhx87@126.com

pISSN 1017-7825, eISSN 1738-8872

Copyright© 2018 by
The Korean Society for Microbiology
and Biotechnology

*Leuconostoc mesenteroides* can be used to produce mannitol by fermentation, but the mannitol productivity is not high. Therefore, in this study we modified the chromosome of *Leuconostoc mesenteroides* by genetic methods to obtain high-yield strains for mannitol production. In this study, gene knock-out strains and gene knock-in strains were constructed by a two-step homologous recombination method. The mannitol productivity of the *pat* gene (which encodes phosphate acetyltransferase) deletion strain (Δ*pat*::*amy*), the *fk* gene (which encodes fructokinase) deletion strain (Δ*fk*::*amy*) and the *stpk* gene (which encodes serine-threonine protein kinase) deletion strain (Δ*stpk*::*amy*) were all increased compared to the wild type, and the productivity of mannitol for each strain was 84.8%, 83.5% and 84.1%, respectively. The mannitol productivity of the *mdh* gene (which encodes mannitol dehydrogenase) knock-in strains (Δ*pat*::*mdh*, Δ*fk*::*mdh* and Δ*stpk*::*mdh*) was increased to a higher level than that of the single-gene deletion strains, and the productivity of mannitol for each was 96.5%, 88% and 93.2%, respectively. The multi-mutant strain Δ*dts*Δ*ldh*Δ*pat*::*mdh*Δ*stpk*::*mdh*Δ*fk*::*mdh* had mannitol productivity of 97.3%. This work shows that multi-gene knock-out and gene knock-in strains have the greatest impact on mannitol production, with mannitol productivity of 97.3% and an increase of 24.7% over wild type. This study used the methods of gene knock-out and gene knock-in to genetically modify the chromosome of *Leuconostoc mesenteroides*. It is of great significance that we increased the ability of Leuconostoc mesenteroides to produce mannitol and revealed its broad development prospects.

**Keywords:** *Leuconostoc mesenteroides*, mannitol productivity, gene knock-out, gene knock-in

## Introduction

Mannitol, a C6 sugar alcohol, is widely used in the chemical, medical and food industries [1]. At present, industrial production of mannitol usually occurs through catalytic hydrogenation [2]. However, this production method requires high pressure at a high temperature with the poor choice of Raney nickel as catalyst [3]. If a 50/50 glucose-fructose mixture is used as the substrate, a 25/75 mannitol-sorbitol mixture will be obtained [4]. Moreover, it is relatively difficult to separate sorbitol and mannitol, which results in even higher production costs and decreased yields [5].

The mannitol content in phaeophyta reaches only as much as 10%–20% and it can be extracted by water recrystallization, ethanol extraction or electrodialysis [6]. Although the extraction method can be used to obtain mannitol, the production cost is relatively high, and the extraction process generates a large amount of waste water and pollutes the environment.

Mannitol can also be produced by enzyme conversion and microbial fermentation [7]. Enzymatic production of mannitol from fructose with mannitol dehydrogenase is also possible [8]. Furthermore, this reaction requires a high-priced co-factor such as NAD(P)H that also needs to be regenerated [9].

It is well known that many microorganisms in nature can synthesize mannitol, such as yeast, mold and some bacteria strains have mannitol production capacity [7]. Tomaszewska, L. *et al.* [10] used the *Yarrowia lipolytica* yeast to produce

mannitol, but the fermentation period is 10 days. The long fermentation period of yeast and mold resulted in lower rate of productivity [6]. Homo-fermentative LAB can produce mannitol, but at very low levels [5]. Mainly hetero-fermentative lactic acid bacteria also produce mannitol without the co-formation of sorbitol [11].

*Leuconostoc mesenteroides*, a group of hetero-fermentative lactic acid bacteria, is a gram-positive bacterium and aerotolerant anaerobe. The chromosome genome of *Leuconostoc mesenteroides* is about 2 M (https://www.ncbi.nlm.nih.gov/genome/- genomes/1078), and the metabolic pathway is uncomplicated and the fermentation period of *Leuconostoc mesenteroides* is shorter than that of yeast [12]. Simplicity of separation and purification of mannitol is a process in which the small, white needle-like crystals of mannitol appeared upon refrigeration of the cell-free fermentation broth at 4°C [13]. Mannitol can be produced (Fig. 1) when *Leuconostoc* are fermented with fructose and glucose [14], or only with fructose [15] or sucrose [16]. In order to reduce fermentation costs, Fontes CP. *et al.* [17] used sucrose as a substrate. However, sucrose, under the action of dextransucrase, can be converted to dextran to reduce mannitol productivity [18]. Therefore, Zhang *et al.* [19] inactivated the *dts* gene which encodes the dextransucrase, resulting in a decrease of dextran in the mutant by 28.8% and an increase in the mannitol yield of 15.3%.

In order to increase mannitol productivity, in this study the *fk*, *pat*, and *stpk* genes (which encode the fructokinase, phosphate acetyltransferase and serine-threonine protein kinase respectively) were knocked out and the *mdh* gene (which encodes the mannitol dehydrogenase) was knocked in.

**Fig. 1.** The main carbon metabolic pathways in *L. mesenteroides*. (1) glucokinase, (2) glucose-6-phosphate dehydrogenase, (3) 6-phosphogluconate dehydrogenase, (4) ribulose-5-phosphate 3-epimerase, (5) phosphoketolase, (6) glyceraldehyde 3-phosphate dehydrogenase, (7) phosphoglycerate kinase, (8) phosphoglycerate mutase, (9) enolase, (10) pyruvate kinase, (11) lactate dehydrogenase, (12) phosphotransacetylase, (13) aldehyde dehydrogenase, (14) alcohol dehydrogenase, (15) acetate kinase, (16) dextransucrase, (17) sucrose phosphorylase, (18) fructokinase, (19) mannitol dehydrogenase, (20) phosphoglucose mutase, (21) phosphoglucose isomerase.

## Materials and Methods

### Microorganisms and Medium

The bacterial strains used in this study are listed in Table 1. *E. coli* DH5α was grown in LB broth or on agar plates at 37°C and supplemented with 100 mg/l ampicillin. *L. mesenteroides* CGMCC1.10327 and *L. mesenteroides* ATCC8293 were grown in MRS medium [19] or on agar plates of MRS at 30°C, with added trypan blue and soluble starch [20].

### PCR Amplification of DNA

The chromosomal DNA of *Leuconostoc* can be extracted by the method of Vingataramin et al [21]. The plasmids and primers used in this study are listed in Tables 2 and 3, respectively.

The *fk* fragment, *pat* fragment and *stpk* fragment were amplified by PCR from *L. mesenteroides* CGMCC1.10327 chromosomal DNA using oligonucleotides pat-u/pat-d, fk-u/fk-d and stpk-u/stpk-d (Table 2) as primers, respectively. The α-amylase expression cassette (α-*amy*) was amplified by PCR from pTA2-amy using oligonucleotides amy-u/amy-d (Table 2) as primers. The *mdh* expression cassette was amplified by PCR from *L. mesenteroides* ATCC8293 chromosomal DNA using oligonucleotides mdh-u/mdh-d (Table 2) as primers. The *fk* fragment, *stpk* fragment and *pat* fragment were digested with the restriction endonuclease (restriction sites were introduced by primers) and inserted into similarly digested pUC19, resulting in pUC19-fk, pUC19-pat and pUC19-stpk, respectively.

**Table 1.** Strains used in this study.

| Strains | Relevant characteristics | Source reference |
|---|---|---|
| *E. coli* DH5α | *supE*44Δ*lacU*169 (φ80 *lacZ*ΔM15) *hdsR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1 | TaKaRa |
| CGMCC1.10327 | Wild type *L. mesenteroides* | This laboratory |
| ATCC 8293 | Wild type *L. mesenteroides* | This laboratory |
| Δ*pat*::*amy* | CGMCC1.10327 with knock-out *pat* and knock-in *amy* | This work |
| Δ*fk*::*amy* | CGMCC1.10327 with knock-out *fk* and knock-in *amy* | This work |
| Δ*stpk*::*amy* | CGMCC1.10327 with knock-out *stpk* and knock-in *amy* | This work |
| Δ*pat*::*mdh* | CGMCC1.10327 with knock-out *pat* and knock-in *mdh* | This work |
| Δ*fk*::*mdh* | CGMCC1.10327 with knock-out *fk* and knock-in *mdh* | This work |
| Δ*stpk*::*mdh* | CGMCC1.10327 with knock-out *stpk* and knock-in *mdh* | This work |
| Δ*stpk* | CGMCC1.10327 with knock-out *stpk* | This work |
| Δ*pat*::*mdh*Δ*stpk*::*mdh* | CGMCC1.10327 with knock-out *pat*, *stpk* and knock-in *mdh* | This work |
| Δ*pat*::*mdh*Δ*fk*::*mdh* | CGMCC1.10327 with knockout *pat*, *fk* and knockin *mdh* | This work |
| Δ*fk*::*mdh*Δ*stpk*::*mdh* | CGMCC1.10327 with knock-out *fk*, *stpk* and knock-in *mdh* | This work |
| Δ*pat*::*mdh*Δ*stpk*::*mdh*-Δ*fk*::*mdh* | CGMCC1.10327 with knock-out *pat*, *stpk*, *fk* and knock-in *mdh* | This work |
| Δ*dts*Δ*ldh*Δ*pat*::*mdh*-Δ*stpk*::*mdh*Δ*fk*::*mdh* | CGMCC1.10327 with knock-out *dts*, *ldh*, *pat*, *stpk*, *fk* and knock-in *mdh* | This work |
| Δ*pat*::*amy*/*pat* | pCW7-*pat* transformated into Δ*pat*::*amy* | This work |
| Δ*fk*::*amy*/*fk* | pCW7-*fk* transformated into Δ*fk*::*amy* | This work |
| Δ*stpk*::*amy*/*stpk* | pCW7-*stpk* transformated into Δ*stpk*::*amy* | This work |

**Construction of Homologous Recombination Vector**

The up-stream and down-stream flanking regions of the gene were amplified by PCR from pUC19-fk, pUC19-pat and pUC19-stpk separately. Both flanking regions were then spliced by overlap extension PCR (KpnI was introduced by primers). These PCR products were digested with EcoRI and HindIII and subsequently ligated to similarly digested pUC19, resulting in homologous recombination plasmids without a marker gene. Then the α-*amy* expression cassette was digested with KpnI and inserted into the homologous recombination plasmids, resulting in homologous recombination plasmids which carried an α-amylase marker gene. In the same way, the *mdh* expression cassette was inserted into the homologous recombination plasmids, resulting in homologous recombination plasmids which carried the *mdh* expression cassette.

**Construction of Mutant Strains and Complement Strains**

Competent cells of *L. mesenteroides* can be prepared by the method of Zhang *et al.* [19]. A fresh culture of strain CGMCC1.10327 was inoculated into MRS broth containing ampicillin (final concentration of 0.48 mg/l), and cells were cultured to reach an $OD_{660}$ of 0.5. Cells were harvested and pre-treated with LiAc-DTT (100 mmol/l LiAc, 10 mmol/l DTT, 0.5 mol/l sucrose, 10 mmol/l Tris-HCl) supplemented with 100 U/ml lysozyme for 20 min. After pre-treatment, PBS (1 mmol/l $KH_2PO_4$-$K_2HPO_4$, 1 mmol/l $MgCl_2$, 0.5 mol/l sucrose) of pH 6.9 was used to wash and resuspend the cells. Next, competent cells were mixed with transforming DNA in a microfuge tube, transferred to cold electroporation cuvettes and placed on ice for 5 min. A pulse was applied under the following condition: 1,400 V, 25 μF, 300 Ω and 4 ms. The cells were immediately resuspended in 1 ml MRS broth containing 2% sucrose and incubated for 3 h at 30°C. The electropulsed strains were diluted $10^7$ on MRS agar plates incubated at 30°C for 5 days, in which trypan blue and soluble starch were added.

In this study, the shuttle vector pCW7 was used, which was constructed by Tian *et al.* [20], to construct the complementary vector. The expression cassettes of *pat*, *fk*, and *stpk* were amplified by PCR from chromosomal DNA of *L. mesenteroides* CGMCC1.10327 respectively. These expression cassettes were then inserted into the shuttle vector pCW7. Afterwards, the complementary vectors were introduced into the single-gene deletion strains by electric transformation.

**Verification of Mutant Strains**

The *pat*, *fk*, and *stpk* fragments were amplified by PCR from the chromosome DNA of single mutant strains and wild-type strains, respectively. The sizes of fragments were compared with a marker by agarose gel electrophoresis.

**Mannitol Assay**

Mutant strains, complement strains and wild-type strains were incubated in liquid fermentation medium. Cultivated for 20 h, the concentrations of mannitol were measured by colorimetric

**Table 2.** Primers used in this study.

| Name | Sequences |
|---|---|
| pat-u | TGTGAATTCTTTTGCTAAGCCTTGT (EcoRI) |
| pat-d | TGTGAATTCGTGAAGATCCCCGTAT (EcoRI) |
| fk-u | ACGAAGCTTGAAGCAGGTGGAACGA (HindIII) |
| fk-d | TGTGAATTCTAGCAACGGGTACGAT (EcoRI) |
| stpk-u | ACGGAATTCTCCTCACTACTTGTTG (EcoRI) |
| stpk-d | ACGGAATTCATGATGCGTCTTGATA (EcoRI) |
| amy-u | TTGGTACCTTTGGCGTGATTATCAG (KpnI) |
| amy-d | TTGGTACCCGAAGGTGAAGTTATAG (KpnI) |
| mdh-u | ATGGTACCATTATGCCTCTTCGCCG (KpnI) |
| mdh-d | ATGGTACCCACGTGATACTGTTGTC (KpnI) |
| patl-u | CGGAATTCTCGACTTATAATGCTTG (EcoRI) |
| patl-d | TAGGATCCTAGGTACCAAGCGAAGAGCGTTATGT (KpnI) |
| patr-u | TTGGTACCTAGGATCCTACTTCGCCTTTTTGCAT (KpnI) |
| part-d | CGAAGCTTAGGCATTTATGGAACTT (HindIII) |
| fkl-u | CGGAATTCTTGCGGATCAC (EcoRI) |
| fkl-d | TTGGTACCGCGCCATTAACGTCAGT (KpnI) |
| fkr-u | AATGGCGCGGTACCAAAAGCATCCT (KpnI) |
| fkr-d | GCAAGCTTCAGCAAAACTT (HindIII) |
| stpkl-u | CAGGAATTCTGTTGAACTGCTTGAGG (EcoRI) |
| stpkl-d | GTCGGATCCGGTACCAGCGTCAGATAGTGTA (KpnI) |
| stpkr-u | GCTGGTACCGGATCCGACCCAACATAATCTC (KpnI) |
| stpkr-d | CGCAAGCTTTGTTGACCGGACACCTA (HindIII) |
| patc-u | GAAGATCTCGATTCCTGTTATCCGCAT (BglII) |
| patc-d | GAAGATCTCGTGGCTTTTTTGGAAGTC (BglII) |
| fkc-u | GAAGATCTCAGATATTATTGAAGTGTT (BglII) |
| fkc-d | GAAGATCTTGAAAATTAAAGTAATGTT (BglII) |
| stpkc-u | GCGTCGACTTTAGACACGTTGTTATTG (SalI) |
| stpkc-d | GCGTCGACTGACGAAAAAGTTGTGATT (SalI) |
| paty-u | ACATTCTCTTCATTGGCTC |
| paty-d | GACTTTATGGAACTTTTTG |
| fky-u | ACTCAGTAGAGCAAGTCAT |
| fky-d | TATCAGGGCGTAAAATCAT |
| stpky-u | GAACTGCTTGAGGAACTAC |
| stpky-d | GACCGGACACCTAATTATG |

determination [19]. To 1 ml of the fermentation supernatant, we added 1.5 ml concentrated hydrochloric acid, and this was heated in a boiling water bath for 10 min (remove the interference of fructose), cooled, diluted, combined with 1 ml sodium periodate solution (0.015 mol sodium periodate dissolved in 0.12 mol/l hydrochloric acid solution) and mixed. After letting the mixture sit at room temperature for 10 min, we added 2 ml 0.1% L-rhamnose solution, and then mixed and added 4 ml freshly prepared Nash reagent (75 g acetic acid, 1 ml glacial acetic acid and 1 ml acetonitrile, dilute to 500 ml). The mixture was heated in a 53°C water bath for 15 min (producing yellow 3, 5- diacetyl-1,4-dehydrodimethylpyridine, which has a large absorption at 412 nm), and cooled to room temperature. The absorbance was measured at 412 nm.

## Results

### Construction of Homologous Recombination Vector

In this study, homologous recombinant vectors were constructed by overlap extension PCR. The up-stream and down-stream flanking regions of the *pat* were amplified by PCR from pUC19-pat, using primer paris patl-u/patl-d, part-u/part-d (KpnI was added to the complementary region of the primer downstream of the left homology fragment and the upstream primer of the right homology fragment), resulting in homologous recombinant vector pUC19-pat. In the same way, it resulted in pUC19-stpk and pUC19-fk too. Then the α-*amy* expression cassette was digested with KpnI and inserted into the homologous recombination vectors, resulting in homologous recombinant vectors pUC19-pat::amy, pUC19-fk::amy and pUC19-stpk::amy, respectively. With the same method, the *mdh* expression cassette was inserted into the homologous recombination vectors, resulting in homologous recombinant vectors pUC19-pat::mdh, pUC19-fk::mdh and pUC19-stpk::mdh, respectively.

### Single Gene Deficiency of *L. mesenteroides*

Acetyl phosphate can be converted into acetyl-coenzyme A to produce ethanol with the phosphate acetyltransferase (*pat*) in PPK pathway [22]. If the *pat* gene is knocked out, it is possible for the procedure to be blocked, and the mixture accumulates more NADH for mannitol production and allows more carbon sources to flow to mannitol production. Jin *et al.* (China patent CN 106754555A) showed that in the *Leuconostoc* genome, there are other genes that function similarly to the acetaldehyde dehydrogenase gene, so there is a portion of ethanol produced when the aldehyde dehydrogenase gene is knocked out. In this study, the homologous recombinant vector pUC19-pat::amy was introduced into *L. mesenteroides* CGMCC1.10327 by the method of electric transformation (Fig. 2A), resulting in the single-gene deletion strain Δ*pat*::*amy*.

Fructose can be converted into 6-phosphoric acid fructose by fructokinase (*fk*), whereupon it then enters the PPK pathway to produce lactic acid and ethanol [23]. The hypothesis of the present work is that inactivation of the *fk* gene would prevent the leakage of fructose into the PPK

**Table 3.** Plasmids used in this study.

| Plasmids | Relevant characteristics | Source or reference |
|---|---|---|
| pUC19 | $Amp^R$ | TaKaRa |
| pUC19-pat | pUC19 containing the gene of *pat* | This work |
| pUC19-fk | pUC19 containing the gene of *fk* | This work |
| pUC19-pat::amy | pUC19 containing α-*amy* upstream and downstream fragment of *pat*, $Amp^R$ | This work |
| pUC19-fk::amy | pUC19 containing α-*amy* upstream and downsteam fragment of *fk*, $Amp^R$ | This work |
| pUC19-stpk::amy | pUC19 containing α-*amy* upsteam and downsteam fragment of *stpk*, $Amp^R$ | This work |
| pUC19-pat::mdh | pUC19 containing *mdh* upsteam and downsteam fragment of *pat*, $Amp^R$ | This work |
| pUC19-fk::mdh | pUC19 containing *mdh* upsteam and downsteam fragment of *fk*, $Amp^R$ | This work |
| pUC19-stpk::mdh | pUC19 containing *mdh* upsteam and downsteam fragment of *stpk*, $Amp^R$ | This work |
| pTA2-amy | pTA2 containing *amy* | The lab |
| pCW7 | pCW4 containing the gene of *apr*, *amy*, and *oriT*, $Amp^R$ | The lab |
| pCW7-fk | pCW7 containing the expression cassettes of *fk*, $Amp^R$ | This work |
| pCW7-pat | pCW7 containing the expression cassettes of *pat*, $Amp^R$ | This work |
| pCW7-stpk | pCW7 containing the expression cassettes of *stpk*, $Amp^R$ | This work |

pathway and give improved yield of mannitol from fructose. In this study, the homologous recombinant vector pUC19-fk::amy was introduced into *L. mesenteroides* CGMCC1.10327 by the method of electric transformation, resulting in the single-gene deletion strain Δ*fk*::*amy*.

Serine threonine protein kinase (*stpk*) is generally considered to exist in the eukaryotic, but it also exists in some bacteria [24]. Proteome analysis of producing dextran and non-producing dextran showed that the association with the two-component system of serine threonine protein kinase was significantly raised when dextran was produced [25]. The hypothesis of the present work is that knock-out of the *stpk* gene would hinder the processing and secretion of dextransucrase, resulting in more conversion of sucrose

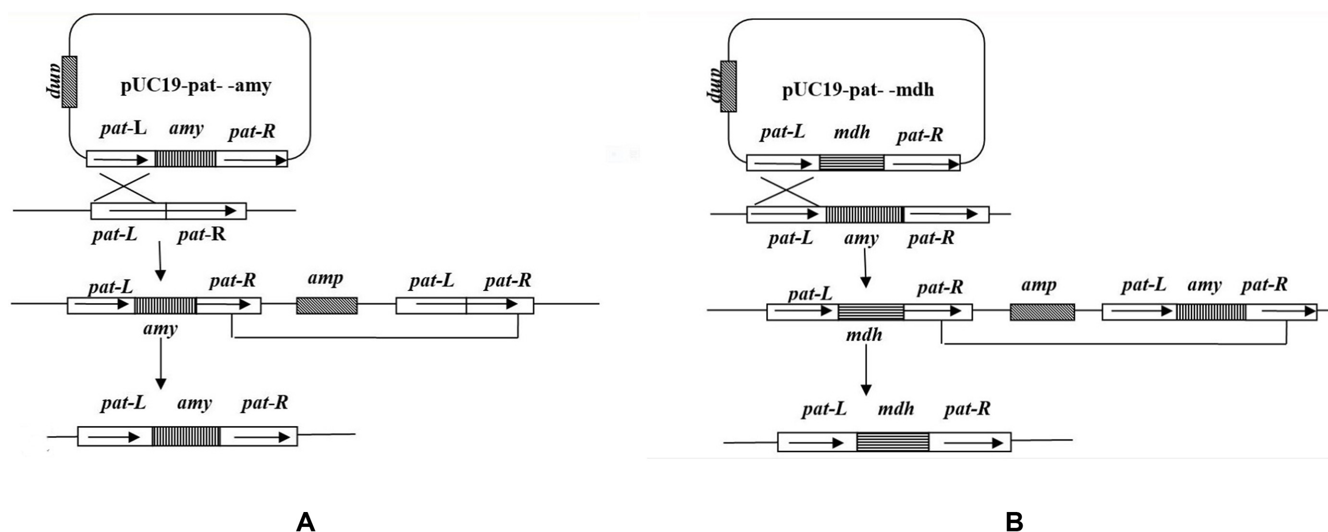


**Fig. 2.** Construction of a disruption in the *L. mesenteroides* pat gene and knock-in mdh gene.

A two-step homologous recombination method was used to knock out the pat gene and knock in the mdh gene. Integration of this plasmid into the chromosome can take place via the pat-L region. This integration results in both a truncated and a disrupted copy of the pat gene. On the other hand, disrupted and intact copies of the gene are obtained when integration occurs via the pat-R region. A: Integration of this homologous recombination fragment which carried the amy gene into the chromosome DNA of *L. mesenteroides*. B: Integration of this homologous recombination fragment which carried the mdh gene into the chromosome DNA of *L. mesenteroides*.

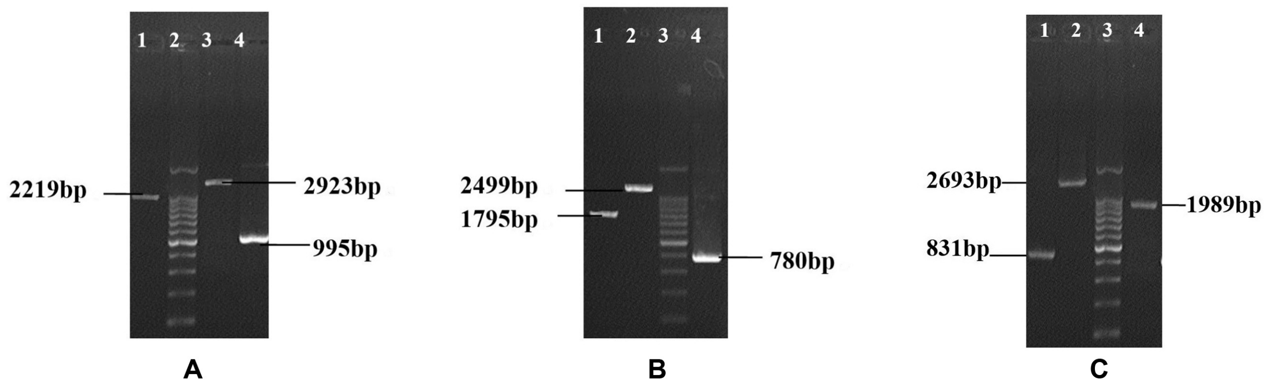


**Fig. 3.** The conformation of pat, fk, stpk inactivation and mdh expression mutant strains by PCR anylasis: M, DNA marker, 1, Δpat::mdh, 2, Δpat::amy, 4, Δfk::mdh, 5, Δfk::amy, 8, Δstpk::amy, 9, Δstpk::mdh, 3, 6, 7, PCR product of pat, fk, stpk, from CGMCC1.10327, respectively.

to fructose, thus increasing mannitol productivity. In this study, the homologous recombinant vector pUC19-stpk::amy was introduced into the *L. mesenteroides* CGMCC1.10327 by the method of electric transformation, resulting in the single gene-deletion strain Δ*stpk*::*amy*.

The single-gene deletion strain Δ*pat*::*amy* was verified by PCR using the primer paty-u/paty-d and the chromosomal DNA of Δ*pat*::*amy* as a template. Similarly, Δ*fk*::*amy* and Δ*stpk*::*amy* were verified by PCR using the primers fky-u/fky-d and stpky-u/stpky-d with the chromosomal DNA of Δ*fk*::*amy* and Δ*stpk*::*amy* as a template, respectively. The sizes of *pat*, *fk* and *stpk* homologous recombination fragments with α-*amy* expression cassette were 2,293 bp, 2,499 bp, and 2,693 bp, respectively. The resultant (Fig. 3) gave the expected fragments in similar PCR tests. Thus, these mutants have the correct structures.

**Effect of Mutants on Mannitol Production**

Yield of fermentation products of mannitol was measured and tabulated (Fig. 4) for batch culture of single-gene deletion strains Δ*pat*::*amy*, Δ*fk*::*amy*, Δ*stpk*::*amy* and wild-type strain. The study found that the accumulation of mannitol reached the maximum when the fermentation period was about 20 h. After 20 h, the mannitol decomposed and was reused, resulting in decreased accumulation of mannitol (Mannitol is weakly used) [26]. The experimental results also showed that the mannitol synthesis ability of each single-gene deletion strain was increased compared with the wild-type strain.

The study showed that the *pat* gene was deleted from a single mutation, resulting in the mannitol productivity reaching 84.8% (an increase of 8.71% over the wild-type strain) and almost no detectable ethanol (ethanol productivity

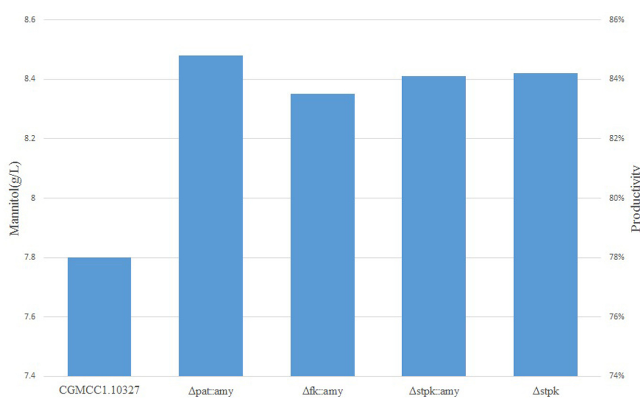


**Fig. 4.** Mannitol (g/l) analysis of sucrose fermentation by wild-type and mutant strains.

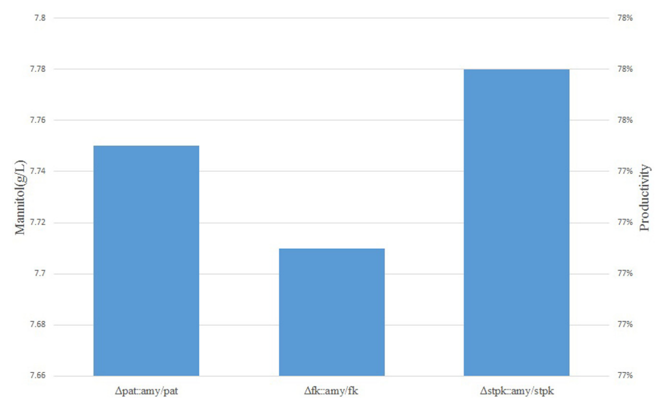


**Fig. 5.** Mannitol (g/l) analysis of sucrose fermentation by wild-type and mutant strains.

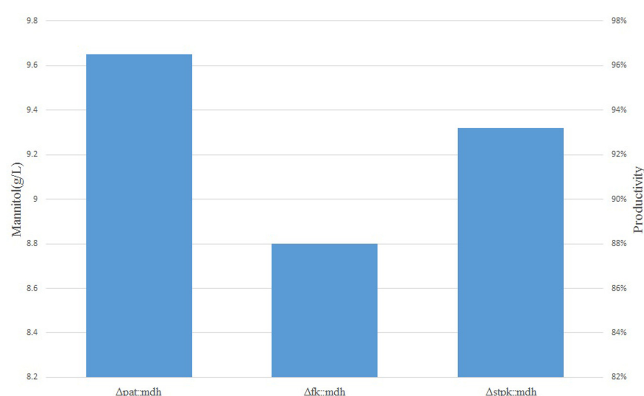


**Fig. 6.** Mannitol (g/l) analysis of sucrose fermentation by wild-type and mutant strains.

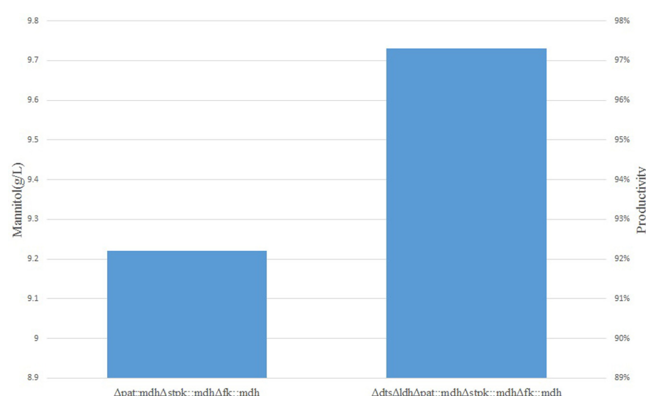


**Fig. 8.** Mannitol (g/l) analysis of sucrose fermentation by wild-type and mutant strains.

is not listed in the Figs. 4−8), so it is far more influential than inactivation of *fk* or *stpk*.

**Construction of Complement Strains**

The shuttle vector pCW7-pat was introduced into the Δ*pat*::*amy* by the method of electric transformation, resulting in complement strain Δ*pat*::*amy*/*pat*. With the same method, the complement strains Δ*fk*::*amy*/*fk* and Δ*stpk*::*amy*/*stpk* were constructed. The yield of the fermentation products of mannitol was measured and tabulated (Fig. 5). There was no significant difference in mannitol production between the complement strains and wild type. It is confirmed that inactivation of *pat*, *fk*, and *stpk* resulted in increased mannitol productivity.

**Knock-in *mdh* of *L. mesenteroides***

*L. mesenteroides* can be used with mannitol dehydrogenase to convert fructose to mannitol. In order to obtain a high-yielding mannitol-producing strain, the *mdh* expression cassette was inserted into the homologous recombination vectors in this study, resulting in pUC19-pat::mdh, pUC19-fk::mdh and pUC19-stpk::mdh, respectively. Then it integrated into the chromosomal DNA of *L. mesenteroides* CGMCC1.10327 by two-step homologous recombination (Fig. 2B).

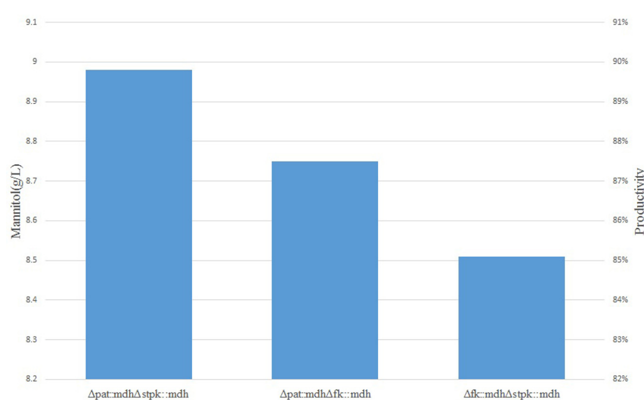


**Fig. 7.** Mannitol (g/l) analysis of sucrose fermentation by wild-type and mutant strains.

The yield of the fermentation products of mannitol was measured and tabulated (Fig. 6). According to this production, the productivity of mannitol was increased so it's better than the single-gene deletion strains. The single-gene-deleted and *mdh* knock-in strain Δ*pat*::*mdh* had the highest mannitol productivity reaching 96.5%, an increase of 23.7% over the wild-type strain.

The knock-in strains were verified by the same method as the single-gene deletion strains. The sizes of *pat*, *fk*, and *stpk* homologous recombination fragments with *mdh* expression cassette were 2,219 bp, 1,795 bp, and 1,989 bp, respectively. The results (Fig. 3) gave the expected fragments as shown in similar PCR tests. Thus, these knock-in strains have the correct structures.

**Double Gene Deficiency and Gene Knock-in of *L. mesenteroides***

In order to further study the effect of phosphate acetyltransferase, fructokinase, serine-threonine protein kinase and mannitol dehydrogenase on the production of mannitol by *L. mesenteroides* CGMCC1.10327, double-gene deficiency and *mdh* gene knock-in strains were constructed. Firstly, the homologous recombinant vector pUC19-fk::amy was introduced into the Δ*pat*::*mdh* by electric transformation, resulting in double-gene deficiency strain Δ*pat*::*mdh*Δ*fk*::*amy*.

Then, the homologous recombinant vectors pUC19-fk::mdh were introduced into the Δ*pat*::*mdh*Δ*fk*::*amy* by electric transformation, resulting in double-gene deficiency and *mdh* gene knock-in strain Δ*pat*::*mdh*Δ*fk*::*mdh*. Similarly, the Δ*pat*::*mdh*Δ*stpk*::*mdh* and Δ*fk*::*mdh*Δ*stpk*::*mdh* were constructed respectively.

The yield of the fermentation products of mannitol was measured and tabulated (Fig. 7). The deficiency of both *pat* and *stpk* in the double-gene deficiency strain was the most significant, and the productivity of mannitol was 89.8% (an increase of 15.1% over the wild-type strain).

**Multi-Gene Deficiency and Gene Knock-in of *L. mesenteroides***

*Leuconostoc* can be used to produce dextran [27] and as lactic acid bacteria, its most important fermentation product is lactic acid. In order to reduce the effect of lactic acid and dextran on mannitol production, Zhang *et al.* [19] knocked out the *dts* (dextransucrase) gene and the mannitol productivity was increased by 15.3% with the wild strain. Tian *et al.* [20] knocked out the *ldh* (lactate dehydrogenase) gene and the mannitol productivity was increased by 7% with the wild strain. In this study, the multi-gene deficiency and *mdh* gene knock-in strains Δ*pat*::*mdh*Δ*stpk*::*mdh*Δ*fk*::*mdh* and Δ*dts*Δ*ldh*Δ*pat*::*mdh*Δ*stpk*::*mdh*Δ*fk*::*mdh* were constructed.

The yield of the fermentation products of mannitol was measured and tabulated (Fig. 8). The experimental results show that the mannitol synthesis ability of multi-gene deficiency and *mdh* gene knock-in strains was increased compared with double-gene deficiency and *mdh* gene knock-in strains. Among these, the highest mannitol productivity was with the multi-gene deficiency and *mdh* gene knock-in strain Δ*dts*Δ*ldh*Δ*pat*::*mdh*Δ*stpk*::*mdh*Δ*fk*::*mdh*, where the mannitol productivity reached 97.3% (increased by 24.7% over the wild strain).

## Discussion

In order to facilitate screening of mutant strains, antibiotic resistance genes are often used as marker genes [28]. To avoid construction of antibiotic-resistant mutant strains, Zhang *et al.* [19] used the overlap extension to splice homologous fragments. Then the culture medium was supplemented with sucrose to screen for strains with low dextran yield for PCR confirmation. However, noantibiotic-resistance results in difficulty when screening for inactivated mutants. To solve this problem, a two-step homologous recombination method was used in this study to obtain markerless gene deficiency and gene knock-in strains. In this method, the α-*amy* expression cassette was used as a marker gene.

Liu X. *et al.* [29] screened on pH 4.5 agar plates with starch as the sole carbon source and observation of the $I_2$-starch clear zone surrounding the colonies. However, $I_2$/KI mix solution cannot be sterilized with the culture medium except being sprayed on the plate, which leads to potential contamination of the strains. The study of Tian *et al.* [20] shows that when soluble starch and trypan blue were added to the agar plate, the *L. mesenteroides* CGMCC1.10327 mutant strain, which carried the α-*amy* expression cassette, would adsorb the trypan blue in the medium with the increase of incubation time, that resulted in the strain becoming blue and the color of the medium becoming lighter. Eric *et al.* [30] isolated the α-*amy* expression cassette from the *Lactobacillus amyloliquefaciens* at a length of 2,862 bp, which has five 273 bp repeats at the 3′ end. However, Eric considered this repetition to be unrelated to amylase activity. Therefore, the α-*amy* expression cassette sequence used in this study was 2,000 bp in length.

The transformants were incubated 3 h, then the strains were diluted $10^7$ on eight agar MRS plates (which included trypan blue and soluble starch), in which the proportion of the target transformant was 1:160. The mutant strains with the α-*amy* marker gene were obtained by electrical transformation with the wild-type strain as receptor bacteria, of which the color of target transformants was blue when incubated for 5 d on agar MRS medium (the color of the wild-type strain is white). The color of the markerless mutant strains and *mdh* knock-in mutant strains was white. It is good to be reminded that strains were obtained by electrical transformation with the mutant strains (which carried the α-*amy* marker gene) as receptor bacteria.

Zhang *et al.* [19] knocked out the *dts* (dextransucrase) gene and the mannitol productivity was increased by 15.3% over the wild strain. Tian *et al.* [20] knocked out the *ldh* (lactate dehydrogenase) gene and the mannitol productivity was increased by 7% over the wild strain. Jin *et al.* (China patent CN 106754555A) knocked out the *aldh* (aldehyde dehydrogenase) gene and the mannitol productivity was increased by 19.8%. In this study, acetate production increased while there was no significant change in lactate production. This result shows that knock-out of the *pat* gene results in the accumulation of acetyle phosphate, thus leading the carbon flux more toward the direction of acetate. This step was also accompanied by the production of ATP, which promotes the growth of the

strain, thereby resulting in a more pronounced mannitol yield increase. This study found that multi-gene knockout has significant effect on mannitol production compared with single-gene knockout. *mdh* gene knock-in increased the productivity of mannitol, of which the highest mannitol productivity was reached with multi-gene deficiency and *mdh* gene knock-in strains (where the mannitol productivity reached 97.3% increased by 24.7% over the wild strain).

In conclusion, whether with gene knock-out or gene knock-in, we have reached the expected hypothesis. The study was based on the redirection of carbons toward the production of byproducts, and led to the development of *Leuconostoc* strains with high efficiency for mannitol production. Therefore, mannitol production was dramatically improved with knock-out of *pat*, *fk*, *stpk* and knock-in of *mdh*. The highest mannitol production was with multi-gene deficiency and *mdh* gene knock-in strain Δ*dts*Δ*ldh*Δ*pat*::*mdh*Δ*stpk*::*mdh*Δ*fk*::*mdh*, where the mannitol productivity reached 97.3% (increased by 24.7% over the wild strains).

## Conflict of Interest

The authors have no financial conflicts of interest to declare.

## References

1. Papagianni M, Legiša M. 2014. Increased mannitol production in *Lactobacillus reuteri* ATCC 55730 production strain with a modified 6-phosphofructo-1-kinase. *J. Biotechnol* **181:** 20-26.
2. Wisselink HW, Weusthuis RA, Eggink G, Hugenholtz J, Grobben GJ. 2002. Mannitol production by lactic acid bacteria: a review. *Int. Dairy J.* **12:** 151-161.
3. Makkee M, Kieboom APG, Van Bekkum H. 1985. Production methods of D-mannitol. *Starch-Stärke* **37:** 136-141.
4. Dai Y, Meng Q, Mu W, Zhang T. 2017. Recent advances in the applications and biotechnological production of mannitol. *J. Funct. Foods* **36:** 404-409.
5. Saha BC, Racine FM. 2011. Biotechnological production of mannitol and its applications. *Appl. Microbiol. Biotechnol.* **89:** 879-891.
6. Wang Y. 2006. The production of mannitol by biotechnics (In Chinese). *Mod. Food Sci. Technol*. **22:** 291-293.
7. Carvalheiro F, Moniz P, Duarte LC, Esteves MP & Gírio FM. 2011. Mannitol production by lactic acid bacteria grown in supplemented carob syrup. *J. Ind. Microbiol. Biotechnol*. **38:** 221-227.
8. Ghoreishi SM, Shahrestani RG. 2009. Innovative strategies for engineering mannitol production. *Trends Food Sci. Technol.* **20:** 263-270.
9. Slatner M, Nagl G, Haltrich D, Kulbe KD, & Nidetzky B. 1998. Enzymatic production of pure D-mannitol at high productivity. *Biocatal. Biotransform.* **16:** 351-363.
10. Tomaszewska L, Rywińska A, Gładkowski W. 2012. Production of erythritol and mannitol by *Yarrowia lipolytica* yeast in media containing glycerol. *J. Ind. Microbiol. Biotechnol*. **39:** 1333-1343.
11. Von Weymarn N, Hujanen M, Leisola M. 2002. Production of D-mannitol by heterofermentative lactic acid bacteria. *Process Biochem.* **37:** 1207-1213.
12. Chung CH. 2006. Production of glucooligosaccharides and mannitol from *Leuconostoc mesenteroides* B-742 fermentation and its separation from byproducts. *J. Microbiol. Biotechnol.* **16:** 325-329.
13. Saha BC, Racine FM. 2008. Production of mannitol by lactic acid bacteria: a review. pp. 391-404. *In* Ching T Hou, Jei-Fu Shaw (eds.), *Biocatalysis and Bioenergy*. Wiley, Hoboken.
14. Song SH, Vieille C. 2009. Recent advances in the biological production of mannitol. *Appl. Microbiol. Biotechnol.* **84:** 55-62.
15. Grobben GJ, Peters SW, Wisselink HW, Weusthuis RA, Hoefnagel MH, Hugenholtz J, *et al*. 2001. Spontaneous formation of a mannitol-producing variant of *Leuconostoc pseudomesenteroides* grown in the presence of fructose. *Appl. Environ. Microbiol.* **67:** 2867-2870.
16. Saha BC. 2006. A low-cost medium for mannitol production by *Lactobacillus intermedius* NRRL B-3693. *Appl. Microbiol. Biotechnol.* **72:** 676-680.
17. Fontes CP, Honorato TL, Rabelo MC, Rodrigues S. 2009. Kinetic study of mannitol production using cashew apple juice as substrate. *Bioprocess Biosyst. Eng*. **32:** 493-499.
18. Yue M, Cao H, Zhang J, Li S, Meng Y, Chen W, *et al*. 2013. Improvement of mannitol production by *Lactobacillus brevis* mutant 3-A5 based on dual-stage pH control and fed-batch fermentations. *World J. Microbiol. Biotechnol.* **29:** 1923-1930.
19. Zhang Z, Cheng WY, Ju XY, Jin HX. 2015. The effect of dextransucrase gene inactivation on mannitol production by *Leuconostoc mesenteroides*. *Indian J. Microbiol*. **55:** 35-40.
20. Tian Y-F, Liu X-L, Cheng W-Y, Jin H-X. 2016. Study on application of α-amylase gene in the gene expression of *Leuconostoc mesenteroides* (In Chinese). *Sci. Technol. Food Ind.* **37:** 203-207.
21. Vingataramin L, Frost EH. 2015. A single protocol for extraction of gDNA from bacteria and yeast. *Biotechniques* **58:** 120-125.
22. Gänzle MG. 2015. Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. *Curr. Opin. Food Sci.* **2:** 106-117.
23. Helanto M, Aarnikunnas J, von Weymarn N, Airaksinen U, Palva A, Leisola M. 2005. Improved mannitol production by a random mutant of *Leuconostoc pseudomesenteroides*. *J. Biotechnol.* **116:** 283-294.
24. Stancik IA, Šestak MS, Ji B, Axelson-Fisk M, Franjevic D, Jers C, *et al*. 2018. Serine/Threonine protein kinases from

bacteria, archaea and eukarya share a common evolutionary origin deeply rooted in the tree of life. *J. Mol. Biol.* **430:** 27-32.

25. Yan M-H, Wu Z-J. 2016. Proteomic analysis of EPS synthesis of *Leuconostoc mesenteroides* (In Chinese). *Sci. Food Technol. Ind*. **37:** 158-160.
26. Chambel L, Chelo IM, Zé-Zé L, Pedro LG, Santos MA, Tenreiro R. 2006. *Leuconostoc pseudoficulneum* sp. nov., isolated from a ripe fig. *Int. J. Syst. Evol. Microbiol.* **56:** 1375-1381.
27. Vidal RF, Martínez A, Moulis C, Escalier P, Morel S, Remaud-Simeon M, *et al*. 2011. A novel dextransucrase is produced by *Leuconostoc citreum* strain B/110-1-2: an isolate used for the industrial production of dextran and dextran derivatives. *J. Ind. Microbiol. Biotechnol.* **38:** 1499-1506.
28. Guo X, Cao C, Wang Y, Li C, Wu M, Chen Y, *et al*. 2014. Effect of the inactivation of lactate dehydrogenase, ethanol dehydrogenase, and phosphotransacetylase on 2, 3-butanediol production in *Klebsiella pneumoniae* strain. *Biotechnol. Biofuels* **7:** 44.
29. Liu X, Jia W, An Y, Cheng K, Wang M, Yang S, *et al*. 2015. Screening, gene cloning, and characterizations of an acid-stable α-amylase. *J. Microbiol. Biotechnol.* **25:** 828-836.
30. Giraud E, Cuny G. 1997. Molecular characterization of the α-amylase genes of *Lactobacillus plantarum* A6 and *Lactobacillus amylovorus* reveals an unusual 3′ end structure with direct tandem repeats and suggests a common evolutionary origin. *Gene.* **198:** 149-157.