SCIREA Journal of Agriculture

http://www.scirea.org/journal/Agriculture

August 12, 2020

Volume 5, Issue 1, February 2020

Establishment of High-throughput Screening System for *Nostoc Flagelliforme* Extracellular polysaccharide (EPS)－producing Recombinant Yeast Strains Based on Duplex PCR Reaction

WenJin Ma[1]*, WenJin Lou[3], Yanbing Zhou[1], Bo Wang[1], Meilin Li[1], Huan Liu[2], XueFeng Chen[2]

[1]The Institute of Gansu Province Light Industrial Scientific Research, Lanzhou 730000, People's Republic of China

[2] School of Food and Biological Engineering, Shaanxi University of Science and Technology, Xi'an 710021, People's Republic of China

[3]Gansu Provincial Academic Industrial for Medical Research, Lanzhou 620100, People's Republic of China

* Corresponding author

Drs. WenJin Ma

The Institute of Gansu Province Light Industrial Scientific Research,

Lanzhou, Gansu 730000 , China

E-mail: mawj0707@163.com.

Abstract

A Common Primer Duplex PCR (CP-D-PCR) was developed to screen polysaccharide-producing recombinant yeast strains from the genotype. This method demonstrated higher sensitivity and efficiency than the conventional methods. In this study, two pairs of primers were designed according to the constitutive protein of photosynthesis from *Nostoc punctiforme* (*N. punctiforme*) PCC73120 which is similar to *Nostoc flagelliforme* (*N.flagelliforme*). Genomic DNA of *N. flagelliforme* and *Rhodotorula mucilaginosa* (*R. mucilaginosa*) were used as templates for amplification and to verify by PCR reaction. Duplex PCR screening system was established on the basis of the simplex PCR screening system and the products of PCR were analyzed by agorae gel electrophoresis. The results showed that both selected primers were able to correctly amplify the objective gene fragment (800 bp, 550 bp) with the *N. flagelliforme* genomic DNA. Electrophoresis showed clear and consistent bands after PCR amplification, respectively. No amplifications were obtained with *R. mucilaginosa* genomic DNA as template. And we have successfully screened five recombinant yeast strains from three hundred and seventy-five strains using duplex PCR screening method. So, the CP-D-PCR was used to screen recombinant yeast strains quickly, accurately and efficiently. This method provides a scientific basis to screen recombinant strains constructed by distant hybridization and no sufficient genetic information about the parent strains.

Keywords: *Nostoc flagelliforme*, *R. mucilaginosa*, Recombinant Yeast Strains, Duplex PCR, Screening System, Gel Electrophoresis

Introduction

N. flagelliforme, *Cyanophyta Nostocacess Nostoc*, is distributed on arid or semiarid steppes of northern and northwestern of China (Gao 1998). It has been used as a kind of food for more than 2,000 years (Tseng 2001). in China, *N. flagelliforme* is favored by Chinese people because of its food and herbal value as well as its pronunciation “Fa cai,” which means “getting rich” in Chinese (Su et al. 2007). EPS of *N. flagelliforme* whose main ingredient is β-type pyran polysaccharide has functions of anti-aging, anti-tumor, hypoglycemic, anticoagulation and so on (Gao 1998; Chen et al. 2010). The pharmacological experiments of

Japan Microalgae Research and Toyama Medical and Pharmaceutical University have shown that EPS has obvious antiviral activity on herpes simplex virus (Kanekiyo et al. 2005). In recent years, the demand for *N. flagelliforme* has increased daily because of edible value of *N. flagelliforme* and medicinal value of EPS. Ever-increasing market demand has led to overexploitation and reduction of *N. flagelliforme* resources, which has resulted in serious damage to vegetation in its growing areas and the deterioration of the environment, causing more frequent sandstorms (You 2000).

Recently, EPS is extracted directly from natural *N. flagelliforme* mainly. Lack of raw material has been the bottleneck of producing EPS commercially because the growing environment of *N. flagelliforme* is special and the technology of artificial culture lags. Therefore, how to produce EPS by official, safe and environmentally friendly method has been a focal point that researchers study. Constructing recombinant yeast strains with the gene of *N. flagelliforme* producing EPS by the DNA recombinant technology is one of the effective methods. It is a new way to produce EPS by culturing recombinant yeast (Yu 2000; Vilaithong et al. 2000). However, discrimination of the recombinant yeast is difficult using conventional methods, based mainly on morphological or metabolites features including culturing the recombinant yeasts in suitable media, extracting EPS with other solvents, and monitoring their presence by chromatographic and other techniques. The limits of these methods are time-consuming and considerable expertise required.

The rapid, sensitive and specific detection that overcomes the disadvantages of conventional method is permitted because of the application of DNA-based techniques, particularly polymerase chain reaction (PCR). PCR-based technology is currently implemented in genetically modified organism (GMO) detection (Lu et al. 2010), disease genotypes of human (Tsakogiannis et al. 2015; Ben M'hadheb et al. 2015) and others (Landete et al. 2010; Apostolidis et al. 2006). And it has been regarded as the most comprehensive approach to recombinant yeast strains detection. PCR methods for detection of recombinant strains can be classified into several types according to the sorts of primers. Primers design for a duplex PCR is a biological problem that requires a combination of databases, tools and methods of data mining. Although the condition for designing primers for simplex PCR is well known, they are not sufficient for the duplex PCR. The process of designing primers for the duplex PCR is more advanced and time consuming. It requires the application of data mining techniques for extensive analysis of larger amount of data coming from various biological databases. Limitations of the complex nature of the duplex PCR process in which several

regions of DNA must be amplified simultaneously and independently should also be considered (Lu et al. 2010). So, in this paper, we designed two specific set of primers to screen EPS-producing recombinant yeast strains. And then a duplex PCR assay set up with two these sets of primers was also successful in detection of recombinant yeast strains. This method is easily carried out, with high throughput, low cost, high specificity, high sensitivity, and is expandable.

Materials and methods

Sample materials

Three different origins of tested *N. flagelliforme* were collected in the eastern side of the Helan Mountain in Yinchuan, Ningxia, China, in 2008, and stored in dry conditions at room temperature for 26 months before used in the experiments. Dissociated and axenic cells were obtained according to previously reported methods (Jia et al. 2005; Su et al. 2007). *N. flagelliforme* was cultured in BG-11 medium .The conditions of culture were 12 h with the light intensity of 60 μmol·m^{-2}·s^{-1}（1000 Lux=15.76 μmol·m^{-2}·s^{-1}）at 25 °C in daytime and 12 h with no light at 10 °C in the evening.

Three different tested *R. mucilaginosa* strains which were used in this study were stored in our lab. *R. mucilaginosa* strains were grown at 37 °C in YPD (yeast extract peptone dextrose) medium containing (g/L) glucose 20, peptone 20, and yeast extract 10 at pH 6.5.

Extraction of genomic DNA

Three *N. flagelliforme* samples were selected as materials for DNA isolation. The method of Saghai-Maroof et al. (Saghai-Maroof et al. 1984) was suitably modified for the isolation of DNA from *N. flagelliforme*. All the steps were performed at room temperature (25 ± 2 °C) unless otherwise mentioned. The main steps in this tactic were as follows: (1) *N. flagelliforme* cells in logarithmic growth phase were centrifuged and washed once by aseptic water, twice with 1.0 ml RS buffer (0.1 M NaCl, 1 mM EDTA, pH 8.0) to reduce EPS, and then twice by aseptic water in steps. Afterwards the cells were collected by centrifuging 10 min at 6000 r·min^{-1}. This step is aimed to eliminate inorganic salt in the medium and the impurity at the cellular surface； (2) The cell walls were broken by the glass homogenizer twice, which is aimed to decrease the amount of lysozyme. Then cells were centrifuged 10 min at 6000 r·min^{-1}; (3) the broken cells were transferred to the centrifugal tube with 3000 μL 2×CTAB

extracting solution (100mM Tris-HCl , 20 mM EDTA, 1.4 M NaCl, 0.2% mercaptoethanol, 2.0% CTAB, pH 8.0) which had been preheated at 65 °C. The mixtures were then incubated in the water bath (65 °C) for 1h and the shaken every 15 min; (4) chloroform and isoamyl alcohol, 24:1 (vol/vol), was added, the tube was shaken gently and centrifuged 10 min at 12000 $r \cdot min^{-1}$. This step was repeated 1-2 times. It can be stopped when there was only water phase and no other phase; (5) The isopropyl alcohol (precooled at -20 °C and volume equaled to 2/3 of the supernatant) was added to the tube. The mixture was then kept for 30 min at -20 °C; (6) The mixture was centrifuged 10 min at 12000 $r \cdot min^{-1}$ at 4 °C. The supernatant was discarded; (7) 720 μL 75% ethanol and 80 μL 1.5 M NaAc were added to the precipitation. The mixture was maintained for 30 min at the room temperature; (8) The mixture was centrifuged 10 min at 8000 $r \cdot min^{-1}$ under 4 °C. The supernatant was discarded. Precipitation was washed 30 min by 800 μL 75% ethanol. And it was centrifuged 10 min at 6000 $r \cdot min^{-1}$ at 4 °C; (9) The precipitation was washed 30 min by 800 μL absolute ethanol. And it was centrifuged 10 min at 6000 $r \cdot min^{-1}$ at 4 °C. The precipitation was dried in air. It can be stopped when there was no ethanol; (10) The DNA was dissolved in 100 μL TE solution which contained 1μL RNase (10 $mg \cdot L^{-1}$), and the mixture was incubated for 30 min at 37 °C. The DNA was preserved at -20 °C for use.

Genomic DNA of the *R. mucilaginosa* strains were extracted using the TIANamp Yeast DNA Kit. Concrete experimental steps were followed the instruction. The TIANamp Yeast DNA Kit was from Tiangen Biotechnology (Beijing) Co., Ltd.

The concentration of DNA isolations was determined from the absorbance at 260 nm and 280 nm on 725-ultraviolet grating spectrophotometer, which were the OD value. The experiments were repeated 3 times. Then the quality of extracted DNA was estimated. Extracted DNA of 6 μL with 2 μL 6×loading buffer which mixed uniformly was tested by 0.8% agorae gel electrophoresis, whose condition was 0.5×TAE buffer, 95 V and 45-55 min. The gels were observed and photographed in the FR-980A biological electrophoresis imaging analysis system (λ=312 nm).

Primer design

Table 1 Information of primer sequences used in this study

Primer		Primer Sequences 5′ -3′	Expected amplicon size (bp)	T_m (°C)
Pho1	F	ACCACCTTCGTCACCTCT	800	51.0
	R	GGATACCCTCGTTCAGCA		
Pho2	F	TAGGAAGAAGCGAAAGCG	550	52.0
	R	CAGTTGATGGAATGGGTG		

***F* forward primer, *R* reverse primer, *bp* base pairs**

Due to the lack of genetic information in the *N. flagelliforme*, selecting primers were designed according to relevant enzyme of photosynthesis of *N. punctiforme* PCC73120 and synthesized by Shanghai Generay Biotechnology Co., Ltd. (The primer information is shown in Table 1). The objective band of primers gene were 800bp (GenBank: ACC82914.1) and 550bp (GenBank: ACC79753.1). Amplitude sizes were selected during the primer design to allow for co-amplification and appropriate separation on agorae gel by electrophoresis to facilitate the unambiguous identification of the PCR products by size. Primers with acceptable physicochemical parameters were selected and aligned with the sequence from the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) to test for possible nonspecific interactions. Primers were further investigated with the use of OligoAnalyzer 1.1.2. Which was used to evaluate critical primer parameters, including the melting temperature (Tm), difference in melting temperatures for primer pairs (ΔTm), GC content (GC%), self-complementarity, repetitive sequences and Gibbs free energy. The PCR products were purified for sequencing by Shanghai Generay Biotechnology Co., Ltd.

PCR set up

Simplex and duplex PCR reactions were performed in a final volume of 25 μl in the MyCycler Thermal Cycler (Bio-Rad).

Simplex reactions system of PCR was template 1.5 μL (70-80 ng), 2×Taq PCRMaster Mix 12.5 μL, every primer 1.0 μL (concentration was 10 μM), and last added to 9 μL ddH_2O. And the program of PCR amplification was that initial degeneration was at 94 °C for 2 min; then followed by 18 cycles each with degeneration at 94 °C for 20 s, anneal at 52 °C for 25 s,

extended at 72 °C for 30 s; at last extended at 72 °C for 2 min.

Duplex PCR reactions system of PCR was template 3.0 μL (140-160 ng), 2×Taq PCRMaster Mix 12.5 μL, every primer 1.5 μL (concentration was 10 μM), and last added to 3.5 μL ddH_2O. And the program of PCR amplification was that initial degeneration was at 94 °C for 2 min; then followed by 20 cycles each with degeneration at 94 °C for 20 s, anneal at 52 °C for 25 s, extended at 72 °C for 30 s; at last extended at 72 °C for 2 min.

PCR products were tested by 1.0% agorae gel electrophoresis, whose condition was 0.5×TAE buffer, 110 V and 40 min. The gels were observed and photographed in the FR-980A biological electrophoresis imaging analysis system (λ=312 nm).

Homology comparison

PCR products were purified for sequencing by Shanghai Generay Biotechnology Co., Ltd. Homology analysis of the deduced amino acid sequences was performed by BLAST and subsequently compared with GenBank data.

Screening for recombinant yeast strains based on PCR reaction

Recombined yeast cells obtained through transferring genomic DNA of *N. flagelliforme* to *R. mucilaginosa* by nitrogen ion implantation. Under the experimental conditions described, yeast strains were implanted using nitrogen ions (N^+) at 20 KeV and a dose of 25×10^{15} ions/cm^2 at a vacuum pressure of 10^{-3} Pa. After ion implantation, the treated cells were immediately placed in 2 mL of 400 μg/mL *N. flagelliforme* genomic DNA in TE buffer, statically incubated for 2 h at 28–30 °C. The eluent was spread evenly on agar plates.

Genomic DNA of recombined strains was extracted using the TIANamp Yeast DNA Kit. Concrete experimental steps were followed the instruction. The TIANamp Yeast DNA Kit was from Tiangen Biotechnology (Beijing) Co., Ltd. Recombined yeast genomic DNA was used as templates for amplification and to verify by simplex and duplex PCR reaction.

Results

Isolation of genomic DNA

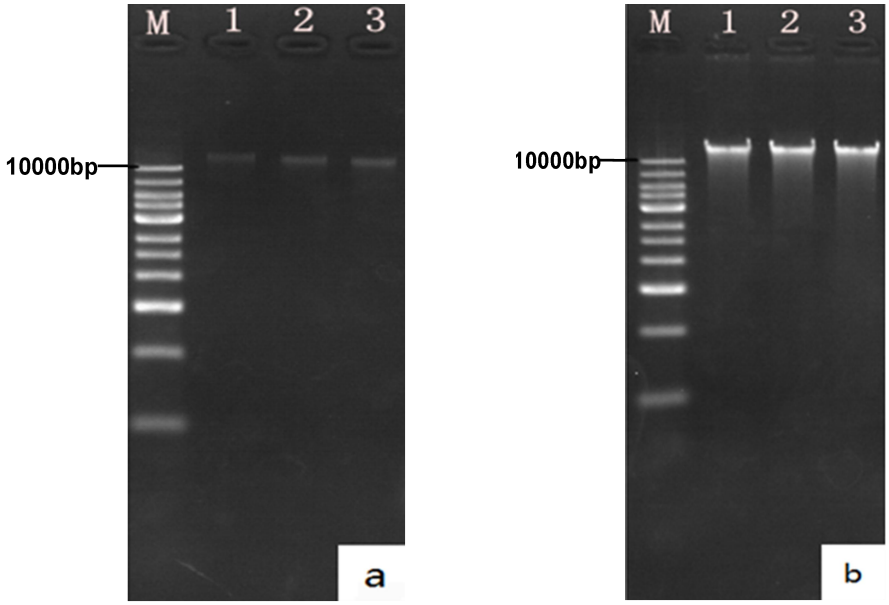

Fig. 1a Agorae gel (0.8%, w/v) electrophoresis (95 V for 45-55 min) of *N. flagelliforme* DNA prepared by modified method. Lanes 1: 10000-bp marker (TIANGEN Biotech Co., Ltd., Beijing, China). The sizes (bp) were indicated on the left. Lanes 2-4: Genomic DNA of *N. flagelliforme* from three different origins. Fig. 1b Agorae gel (0.8%, w/v) electrophoresis (95 V for 45-55 min) of *R. mucilaginosa* strains by TIANamp Yeast DNA Kit. Lanes 1: 10000-bp marker (TIANGEN Biotech Co., Ltd., Beijing, China). The sizes (bp) were indicated on the left. Lanes 2-4: Genomic DNA of *R. mucilaginosa* strains from three different origins.

Fig. 1a showed the results of three extracted genomic DNA from *N. flagelliforme* by our method. There were no tailing phenomenons, indicating that the quality of DNA extracted by our method was good and the DNA was complete. Ratios of OD260nm/OD280nm were about 1.8, which showed that the purity was high (Table 2).

Table 2 D (λ) value and content of DNA from *N. flagelliforme* and *R. mucilaginosa*

Serial number	OD_{260}	OD_{280}	OD_{260}/OD_{280}	Concentration（μg/mL）
N. flagelliforme strains				
1	0.078±0.004	0.042±0.003	1.822	3.90
2	0.063±0.002	0.035±0.001	1.806	3.15
3	0.076±0.009	0.043±0.004	1.809	3.80
R. mucilaginosa strains				

4	0.123±0.003	0.065±0.003	1.8923	6.15
5	0.131±0.002	0.072±0.001	1.8194	6.55
6	0.126±0.003	0.068±0.004	1.8529	6.30

Values are the means ± standard deviation (n = 3)

Fig. 1b showed the results of three extracted DNA from *R. mucilaginosa* stains by the TIANamp Yeast DNA Kit. The quality of genomic DNA with the OD260nm/OD280nm ratio of 1.8, and the gel electrophoresis analysis revealed high quality and high yield of genomic DNA extracted (Table 2).

Primer design

The constitutive protein of photosynthesis genes from *N. punctiforme* PCC73120 was selected as the target genes, and suitable primers were designed to detect recombinant yeast strains. The following general requirements for design were considered: the primers have similar Tm values; the primers would not form hairpins, homodimers or heterodimers or have repetitive sequences; and the target fragment gap should exceed 30 bp to meet the resolution requirements for agorae gel electrophoresis. Two pairs of primers were designed with similar physical characteristics to allow for simultaneous amplification under the same conditions in single or multiplexed reactions (Aguilera-Arreola et al. 2014). The lengths of the primers were 18 bp, and their melting temperatures were between 51 °C and 52 °C. These characteristics are important because longer primers allowed the reaction to be performed at a higher annealing temperature and yielded fewer nonspecific products. No undesirable primer-primer interactions or repetitive sequences were detected. The expected amplicon sizes were 800 bp and 550 bp, respectively.

Development of Simplex PCR assay

The newly designed primers successfully detected *N. flagelliforme* sources, respectively. The amounts of primers pho1 and pho2 was 1.0 μL (concentration was 10 μM), respectively. For pho1 templates the optimal annealing temperature was 51 °C, the other was 52 °C. And the program of PCR amplification cycles was 18.

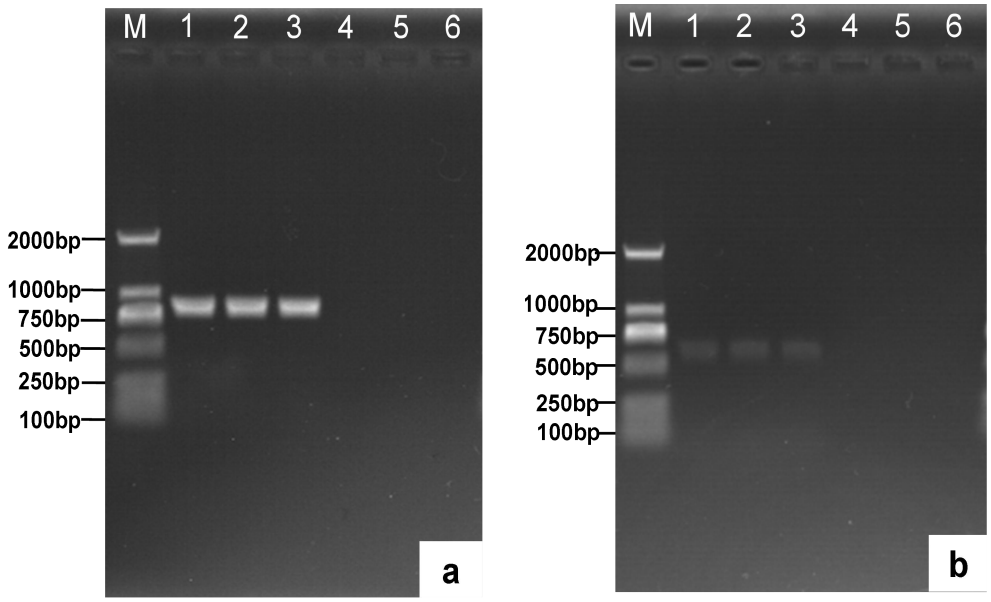

Fig. 2a Specificity of simplex PCR of Pho1. Lanes 1: 2000-bp marker (TIANGEN Biotech Co., Ltd., Beijing, China). The sizes (bp) were indicated on the left. Lanes 2-4: Three different origins of *N. flagelliforme*. Lanes 5-7: Three different origins of *R. mucilaginosa*. Fig. 2b Specificity of simplex PCR of Pho2. Lanes 1: 2000-bp marker (TIANGEN Biotech Co., Ltd., Beijing, China). The sizes (bp) were indicated on the left. Lanes 2-4: Three different origins of *N. flagelliforme*. Lanes 5-7: Three different origins of *R. mucilaginosa* strains.

Three samples for genomic DNA extraction from *N. flagelliformes* and *R. mucilaginosa* were used as the template for PCR simplex verification, respectively. Fig. 2 showed that both selected primers were able to correctly amplify the objective gene fragment (800bp, 550bp) with the *N. flagelliforme* DNA in lanes 2-4. And gel electrophoresis of the simplex PCR products showed a clear signal of amplification, respectively. Fig. 2 showed no amplifications were obtained from another three samples which took *R. mucilaginosa* DNA as template in lanes 5-7, respectively.

Development of duplex PCR assay

The optimized duplex PCR conditions included the annealing temperature of 52 °C and the following system of PCR: template 3.0 μL (140-160 ng), every primer was 1.5 μL (concentration was 10 μM). And the program of PCR amplification cycles was 20.

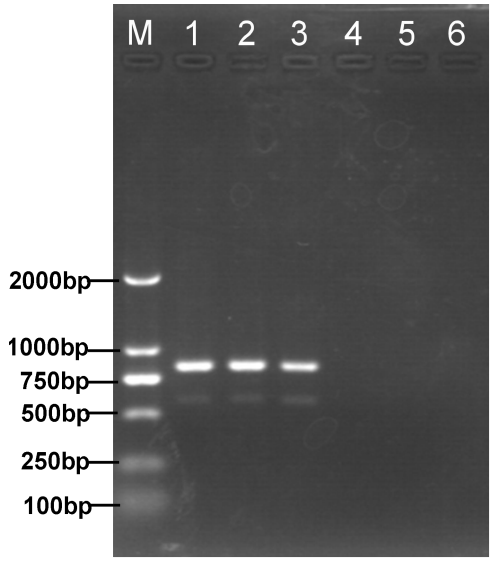

Fig. 3 Specificity of duplex PCR of pho1 and pho2. Lanes 1: 2000-bp marker. The sizes (bp) were indicated on the left. Lanes 2-4: Three different origins of *N. flagelliforme*. Lanes 5-7: Three different origins of *R. mucilaginosa*.

Fig. 3 showed that no significant changes were observed when each single *N. flagelliforme* DNA template was detected using the reaction mix containing duplex primers sets, indicating that no interference occurred between the primers sets. Both selected primers were able to clearly amplify targets with the *N. flagelliforme* DNA in lanes 2-4. No amplifications were obtained with *R. mucilaginosa* DNA from lanes 5-7 by the specific primers.

Therefore, established duplex PCR screening system can be applied to research on high-throughput screening of EPS-producing recombinant yeast strains.

Homology comparison

PCR products were purified for sequencing and the deduced amino acid sequences was performed by BLAST and subsequently compared with GenBank data. The results showed that the full length of *Pho1* was about 800bp and the other was 550bp. The sequences shared high homology with specific genes of *Nostoc sp.* PCC7524, *Nostoc sp.* PCC7107, *Nostoc sp.* PCC7120 were 87%、86%、86%, respectively.

Discussion

N. flagelliforme is a terrestrial macroscopic filamentous cyanobacterium that is distributed on some arid and semi-arid areas of China, and its herbal value was recognized more than 400 years ago (Gao 1998). EPS of *N. flagelliforme* has been proved to possess the properties of

antivirus, antioxidant, and anti-tumor (Chen et al. 2010). In recent years, EPS is extracted directly from natural *N. flagelliforme* mainly. Lack of raw material and the lag of artificial culture technology have been bottleneck of producing EPS commercially. To produce EPS by recombinant yeast strains is one of the effective methods. But it is very difficult to find recombinant yeast strains from the starting strains because of the heavy workload. Most researchers continued to use traditional method to screen recombinant yeast strains. Lü et al. (Lü et al. 2009) has randomly transferred the genomic DNA from *Ephedra glauca* to *Saccharomyces cerevisiae* and *Hansenula anomala* by argon and nitrogen ion implantation. Through repeated sub-culturing and using reversed phase high-performance liquid chromatography analysis to quantify the concentrations of the secondary metabolites, l-ephedrine and d-pseudoephedrine, 12 recombinant strains of genetically stable yeast were obtained. Qian et al. (Qian et al. 2014) has randomly transferred the genomic DNA from plant *Gentiana macrophylla* into *Hansenula polymorpha* by 25 Kev nitrogen ions (N^+) at a dose of 25×10^{15} ions·cm^{-2} at a vacuum pressure of 10^{-3} Pa. One potential stable recombinant yeast strain capable of producing *Gentiopicroside* was obtained using a combination screening of Fehliing ' s test and TLC method. The use of conventional methods based mainly on morphological or metabolites' features to distinguish recombinant yeast strains are often not performed immediately, and it could take more than three days for the results to become available, and considerable expertise required. How to design experiments and seek a convenient and efficient method are the key challenges researchers facing.

A large number of molecular techniques have been developed in order to distinguish recombinant strains. PCR is the most prevalent method with sensitive and specific for yeast typing, especially with the use of specific primers that amplify a broad spectrum of different foreign genotypes. Specific PCR primers have been employed to confirm the presence or absence of target microorganisms or specific features associated with them. The specificity of the primer combinations was demonstrated by the negative PCR results obtained with all of the non-Pho reference stains because there was no amplification in the PCR reaction without photosynthesis genes. In this study, we have developed a duplex PCR assay based on two specific set of primers (the data were shown in the Table 1) designed according to relevant enzyme gene of photosynthesis of *N. punctiforme* PCC73120. Adapter primers were used in simplex and duplex PCR system. The design of primers is very important because the specificity and melting temperature of the primers are specific.

In this study, we have developed a duplex PCR assay to screen recombinant yeast strains.

Duplex PCR is more repeatable, time-saving and affordable than the other mentioned methods (Dalmasso et al. 2004). D-PCR is a rapid tool that allows for the simultaneous amplification of more than one sequence of target DNA in a single reaction, saving time and reagents, thereby overcoming the weakness of simplex PCR detection, which only amplifies a pair of primers. However, duplex PCR is a complex process which has several disadvantages. Which have low amplification efficiency, variable efficiency on different templates and poor universality, highlight the need for an advanced duplex approach. The other advantage of this assay is that it is cheaper and easier than a prode-based assay and RT-PCR assay, with similar higher specificity. In our paper, simplex and duplex PCR reactions were performed in a final volume of 25 μl. Simplex reactions system of PCR was template 1.5 μL (70-80 ng), every primer 1.0 μL (concentration was 10 μM), and the program for PCR amplification was 18 cycles. But Duplex reactions system of PCR was template 3.0 μL (140-160 ng), every primer 1.5 μL (concentration was 10 μM), and the program for PCR amplificat- ion was 20 cycles. Through these methods, finally we could see that no significant changes were observed when each single *N. flagelliforme* DNA template was detected using the reaction mix containing duplex primers sets (Fig. 3) , which indicated that no interference occurred between the primers sets. Both selected primers were able to clearly amplify targets with the *N. flagelliforme* DNA in lanes 2-4. No amplifications were obtained with *R. mucilaginosa* genomic DNA from lanes 5-7 by the specific primers. By sequencing, we obtained the full length of Pho1 was about 800bp and Pho2 was 550bp. As was shown in Fig. 4, five recombinant yeast strains were screened from three hundred and seventy-five strains using the high-throughput screening system based on duplex PCR reaction. These results increase our interest in generating a rapid, inexpensive and practical new methodology that is applicable to a larger number of recombinant yeast strains in a short time and is not dependent on sophisticated technology.

Conclusions

We have developed a method (CP-D-PCR) based on duplex PCR reaction that can successfully identify EPS-producing recombinant yeast strains simply by checking the result of PCR reaction. This method could be fairly easy and more efficient than the conventional detection procedures previously performed. The application of the proposed method could bring innovative perspectives in studies about the molecular identification of the two involved species. Through such methods, the EPS-producing recombinant yeast strains were achieved.

Acknowledgement

This work was supported by key talents project of Gansu Province (Grant No. 2020RCXM078)

References

[1] Aguilera-Arreola MG, González-Cardel AM, Tenorio AM, Curiel-Quezada E, Castro-Escarpulli G (2014) Highly specific and efficient primers for in-house multiplex PCR detection of Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplasma hominis and Ureaplasma urealyticum. BMC research notes 7 (1):433

[2] Apostolidis AP, Apostolou PK, Georgiadis A, Sandaltzopoulos R (2006) Rapid identification of Salmo trutta lineages by multiplex PCR utilizing primers tailored to discriminate single nucleotide polymorphisms (SNPs) of the mitochondrial control region. Conservation Genetics 8 (5):1025-1028. doi:10.1007/s10592-006-9239-1

[3] Ben M'hadheb M, Harrabi M, Souii A, Jrad-Battikh N, Gharbi J (2015) Multiplex RT-PCR and indirect immunofluorescence assays for detection and subtyping of human influenza virus in Tunisia. Current microbiology 70 (3):324-329. doi:10.1007/s00284-014-0719-0

[4] Chen X, Jia S, Yue S, Wang N, Li C, Wang Y (2010) Effect of solid bed-materials on vegetative cells of Nostoc flagelliforme. Journal of applied phycology 22 (3):341-347

[5] Dalmasso A, Fontanella E, Piatti P, Civera T, Rosati S, Bottero M (2004) A multiplex PCR assay for the identification of animal species in feedstuffs. Molecular and Cellular Probes 18 (2):81-87

[6] Gao K (1998) Chinese studies on the edible blue-green alga, Nostoc flagelliforme: a review. Journal of Applied Phycology 10 (1):37-49

[7] Jia S-R, Su J-Y, Qiao C-S (2005) Nostoc flagelliforme cells cultivation and its products. Chinese patent ZL031191010

[8] Kanekiyo K, Lee J-B, Hayashi K, Takenaka H, Hayakawa Y, Endo S, Hayashi T (2005) Isolation of an Antiviral Polysaccharide, Nostoflan, from a Terrestrial Cyanobacterium, Nostoc f lagelliforme. Journal of natural products 68 (7):1037-1041

[9] Lü J, Jin X, Mao P-H, Ma X-D, Ling H-Q, Fan Y-H, Yu L, Wu B-S, Ouyang P-K (2009) Transfer of Ephedra genomic DNA to yeasts by ion implantation. Applied biochemistry

and biotechnology 158 (3):571-581

[10] Landete JM, de las Rivas B, Marcobal A, Muñoz R (2010) PCR methods for the detection of biogenic amine-producing bacteria on wine. Annals of Microbiology 61 (1):159-166. doi:10.1007/s13213-010-0068-6

[11] Lu I-J, Lin C-H, Pan T-M (2010) Establishment of a system based on universal multiplex-PCR for screening genetically modified crops. Anal Bioanal Chen 396:2055-2064

[12] Qian W-D, Zhao D-Z, Fu Y-F, Mao P-H, Zhou Y-X (2014) The construction of Gentiopicroside-producing recombinant yeast using one-step recombinant method. Journal of Shaanxi University of Science & Technology 5 (32):123-128.(in Chinese with English abstract)

[13] Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard R (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proceedings of the National Academy of Sciences 81 (24):8014-8018

[14] Su J, Jia S, Chen X, Yu H (2007) Morphology, cell growth, and polysaccharide production of Nostoc flagelliforme in liquid suspension culture at different agitation rates. Journal of Applied Phycology 20 (3):213-217. doi:10.1007/s10811-007-9221-4

[15] Tsakogiannis D, Diamantidou V, Toska E, Kyriakopoulou Z, Dimitriou TG, Ruether IG, Gortsilas P, Markoulatos P (2015) Multiplex PCR assay for the rapid identification of human papillomavirus genotypes 16, 18, 45, 35, 66, 33, 51, 58, and 31 in clinical samples. Archives of virology 160 (1):207-214. doi:10.1007/s00705-014-2261-6

[16] Tseng C (2001) Algal biotechnology industries and research activities in China. Journal of Applied Phycology 13 (4):375-380

[17] Vilaithong T, Yu L, Alisi C, Phanchaisri B, Apavatjrut P, Anuntalabhochai S (2000) A study of low-energy ion beam effects on outer plant cell structure for exogenous macromolecule transferring. Surf Coat Tech 128:133-138

[18] You Y (2000) Impact of the Nostoc flagelliforme trade on the ecoenvironment of the Inner Mongolia pasture and some policy suggestions. Arid Land Geogr 23 (1):279–283

[19] Yu Z (2000) Ion beam application in genetic modification. Plasma Science, IEEE Transactions on 28 (1):128-132