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Research Article

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Effects on gene expression of yeast *Hansenula anomala* by low-energy nitrogen ion implantation

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ABSTRACT

The original yeast strain, Hansenula anomala 2340, was implanted by low-energy nitrogen ion and the mutant library was obtained. Thin layer chromatography (TLC) was used to detect the newly generated red substance produced by mutant strain N6076 and to evaluate the change of metabolic pathways. In order to explore the change in metabolic pathways of mutant strain, a suppression subtractive hybridization (SSH) technology was used to construct a forward subtractive cDNA library of mutant strain N6076. Through sequencing analysis, a total of 14 gene fragment sequences with high expression levels were obtained, which included 9 differentially expressed genes. Among these genes, 7 differentially expressed genes revealed the mutation in bases. Meanwhile, among these 7 differentially expressed genes, 4 genes with base mutation resulted in the change of protein sequences. In addition, the base deletion was also observed in one gene fragment sequence.

Key words: Gene expression; SSH; Ion implantation; Yeast; Mutant

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INTRODUCTION

Since Hermann Muller proved that X-ray radiation could increase the mutation rate of organisms, radiation mutagenesis technique has been gradually applied to mutation breeding of organisms [1]. On the basis of Muller's innovative works, a variety of radiation mutagenesis sources such as X-ray, gamma-ray, ultraviolet light, laser and electronic sources have been developed and successfully applied in biological breeding with a great deal of achievements [2]. Inspired by these works, in 1988, low-energy ion mutagenesis source has been successfully applied to the breeding of rice in Key Laboratory of Ion Beam Bioengineering at the Institute of Plasma Physics, Chinese Academy of Sciences, which was the first report that ion beam irradiation into rice could produce mutagenic effect [3]. Therefore, a new interdisciplinary research field, Ion Beam Bioengineering, has gained widespread attention [4].

Low-energy ion, as a new source of radiation mutagenesis, is different from traditional radiation mutagenesis source. Low-energy ion can integrate energy deposition, mass deposition, momentum exchange and charge exchange into one so that it is characteristics of easily control dose, less damage, a broad spectrum of mutation, high mutation rate and obvious mutagenic effects [5]. Multiple factor effect produced by low-energy ion irradiation can lead to

complex biological effects in organisms, which is the reason for producing high mutation rate and wide mutation spectrum of organisms. Low-energy ion irradiation technology as a novel genetic modification method is first used for industrial microorganisms and agricultural breeding in China with a series of achievements [6-10]. Currently, the application of this mutation technology in plant breeding has gained tremendous attention. For example, the obvious mutagenic effect during the breeding of buckwheat [11], rose [12], and canation [13] was achieved.

Low-energy ion irradiation technology has gone through 30 years and formed its unique theoretical system. However, the mechanisms of low-energy ion irradiation mutagenesis are still unclear [14-17]. Previous studies of mutagenic materials are not model organisms and lack the necessary sequence information for supporting the research. In the present study, the yeast strain, *Hansenula anomala* 2340, was used as the original material to conduct low-energy ion irradiation and construct the subtractive library of the mutant strains. From the mutant library, a mutant strain N6076 that can produce red substance in the fermentation broth has been screened. In order to uncover the mechanisms of red substance accumulation in the mutant strain, a gene subtractive library has been constructed on the basis of mutant and original strains with high expression through suppression subtractive hybridization (SSH) technology to explore the change of gene expression profile in mutant strains and analyze the generation mechanisms of new red substance.

EXPERIMENTAL SECTION

Yeast strain preparation and low-energy ion irradiation

The original yeast strain, *Hansenula anomala* 2340, was obtained from the Chinese General Microbiological Culture Collection Center. The yeast strain was transferred from slant medium into YPD liquid medium [18], followed by culturing on a rotary shaker at 220 r/min for 12 h at 28-30 °C. The fermentation broth was diluted to 1.0×10^7 CFU/mL with protection solution [19], and 0.1 mL of diluted solution was spread in the middle of a sterile 90-mm petri dish with a sterile glass spatula and air dried under aseptic conditions.

The prepared yeast cell films were placed on the aseptic sample holder in the vacuum target chamber of the ion irradiation facility (Institute of Plasma Physics, Chinese Academy of Sciences). The films were irradiated using nitrogen ions (N^+) at 15 KeV and a dose of 15×10^{15} ions/cm^2 at a vacuum pressure of 10^{-3} Pa. A yeast cell film was treated under vacuum conditions without irradiation of N^+ as the control.

Survival yeast and control cell culture

After low-energy ion irradiation, the treated yeast cell film and the negative control were immediately eluted by YPD liquid medium using a sterile glass spatula for 2 min. Then the eluent was collected and 0.1 mL of eluant was spread on YPD agar plates. The plates were inverted and cultured for 72 h at 28-30 °C. The survival yeast cells grown on YPD agar plates were inoculated into test tubes, cultured at 28-30 °C for 72 h, then inoculated into liquid medium and grown at 28-30 °C for another 96 h with continuous shaking at 230 r/min. At the end of incubation period, yeast cells were removed from the fermentation broth by centrifugation at 8,000 ×g for 10 min. The supernatant of different survival yeast cells and the control were collected for the subsequent analysis.

Yeast mutant isolation and characterization

Mutant yeasts were inoculated in YPD medium with shaking speed of 230 r/min at 28-30 °C for 96 h cultivation. After liquid culture, the culture was centrifuged at 8,000 ×g for 10 min and the supernatant was collected. An equal volume of chloroform was added to the harvested supernatant and the mixture was shaken vigorously for 1 min. The chloroform extract was stored for future use. The control strain extract was prepared using the same method. Meanwhile, silica gel GF_{254} was mixed with 3 volume of 0.5% CMC-Na solution and grinded in a mortar into a paste. After removing the bubbles, the paste was quickly and evenly spread on 150 mm × 200 mm glass plates through oscillation. The silica gel plates were activated at 110 °C for 30 min and kept in a desiccator for future use. A straight line was draw at one end of 2 cm from the bottom of the silica gel plate as the starting line. The chloroform extract was absorbed by a capillary tube and spotted on prepared silica gel plate. The chloroform extract of control strain with 96 h cultivation was used as the control. The loading amount of each sample was 200 μg. The silica gel plate with loaded samples was placed in chromatographic tank containing the developing agent composed of toluene: methyl formate: formic acid at the ratio of 5:4:1 and then subjected to saturation for 15 min. Following the saturation, the end of silica gel plate with loaded samples was immersed in the developing agent for the development in an upward direction. The development was terminated when the front line of developing agent was up to 10 cm. The plate was taken out from the chromatographic tank and dried at room temperature. Red spots were observed under the visible light and the locations of the red component were recorded.

RNA extraction, reverse transcription and double-stranded cDNA synthesis of yeasts

Original and genetically modified yeasts were transformed into liquid medium for 96 h culture at the shaking speed

of 230 r/min and culture temperature of 28-30 °C. The cell pellets were harvested by 12000 ×g centrifugation for 2 min. The strain pellets was treated with liquid nitrogen and grinded into paste. The yeast paste was transferred to DEPC (Takara Company) treated EP tubes. Total RNA was extracted using RNA extraction kit (Takara Company) according to the manufacturer's instruction. The purity and concentration of total RNA were evaluated by 1% agarose gel electrophoresis and UV-754 spectrophotometer.

In addition, the reverse transcription and double-stranded cDNA synthesis of original and genetically modified yeasts were conducted using SMART^{TM} PCR cDNA Synthesis Kit (Clontech Company) according to the manufacturer's manual.

Construction and screening of forward subtractive library of mutant strains

The construction of forward subtractive library was performed using mutant strains as the experimental group and original strains as the driving group through PCR-Select^{TM} cDNA subtraction Kit (Clontech Company) according to the manufacturer's instruction. The second suppression PCR amplified products were harvested and then ligated into PCR2.1 T-vector (Invitrogen, USA). The constructed plasmid was transformed into *Escherichia coli* DH5α (Invitrogen, USA). The mutant strains were screened by blue-white bands to evaluate the construction of forward SSH subtractive library of yeasts.

The N1 and N2 in PCR-Select^{TM} cDNA subtraction Kit were used as the primers and SSH subtractive cDNA library plasmid DNA (diluted 50 fold) was used as the template for PCR amplification. The amplified products were examined by 1% agarose gel electrophoresis and sequence analysis in Shanghai Invitrogen Biotechnology Company (Invitrogen).

Bioinformatic analysis of genes with different expression levels

The homology comparison between differentially expressed gene fragment sequences that were confirmed by sequence analysis and genes from NCBI database (www.ncbi.nlm.nih.gov/blast) were conducted by BLAST method to obtain the basic information of differentially expressed genes. Meanwhile, the genes achieved by homology comparison were future searched in SGD (www.yeastgenome.org) database [19].

RESULTS

Characterization of yeast mutants by thin layer chromatography

The original yeast strain and mutant strain subjected to ion beam irradiation were conducted to fermentation and the fermentation broth was evaluated by thin layer chromatography. As shown in Figure 1, a new red substance generated from the yeast mutant strain N6076 subjected to ion beam irradiation was observed in the thin layer plate with the R_f value of 0.51 under the visible light. In contrast, no band at the location with same R_f value was observed in the control strain, blank culture medium and the survival strain after ion irradiation. These results indicated that this red substance should be a new metabolic product from yeast mutant strain N6076 after ion beam irradiation due to the change of metabolic pathways of the mutant strain.

Fig. 1 Thin layer chromatography (TLC) of (a) yeast fermentation broth, (b) chloroform extract from the fermentation broth of mutant strain N6076 and (c) chloroform extract from the fermentation broth of original yeast strain

Sequencing results of subtractive cDNA library

A total of 384 clones were obtained through the screening of forward subtractive cDNA library of yeast mutant strain established by PCR-Select^{TM} cDNA Subtraction Kit. The PCR amplified products were evaluated by agarose gel electrophoresis. As shown in Figure 2, the gene fragments obtained by SSH technology was in the range of 100-500 bp. Meanwhile, these PCR amplified fragments with various sizes were subjected to sequence analysis.

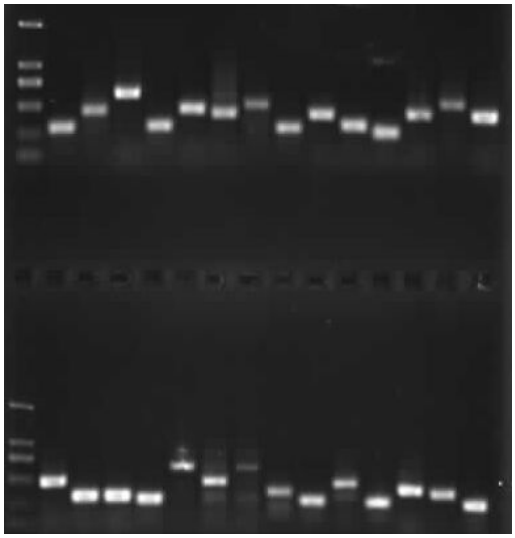

Fig. 2 Identification of PCR amplified fragments from yeast mutant strain through agarose gel electrophoresis

The differentially expressed gene fragment sequences were subjected to BLAST homology comparison in NCBI database. After the removal of low mass gene fragment sequences, repeat gene sequences and mRNA with polyA sequence at 3' end, a total of 14 gene fragment sequences with high expression from the subtractive library of yeast mutant strain N6076 were achieved. Among these 14 gene fragments, 9 gene fragments were differentially expressed genes (Table 1). Meanwhile, several genes related to bioinformation transcription and translation, such as Spr6p (TnsA endonuclease C terminal), Rim4p (Adenylate cyclase NT domain), Rsa3p (ribosome assembly protein 3) and Bmh1p (cell cycle regulated protein), were obtained from highly expressed fragments of yeast mutant strain N6076. These results suggested that the transcript and translation of genetic information was still relatively active after 96 h cultivation of yeast mutant strain N6076. Hxt5p is an inducible enzyme at the condition of non-culture medium with the addition of carbon source, while Agp3p is a highly expressed enzyme in the deficiency of nitrogen source. Both enzymes were highly expressed after 96 h cultivation and resulted in the deficiency of carbon and nitrogen, which could induce the production of enzymes to maintain the energy requirements of normal growth.

Table 1 Comparison of differentially expressed gene fragments

Gene Number	Length	Registered gene	Registered gene length ratio	Base mutation status	Amino acid mutation status
1	142	Spc19p	142/142	No	No
2	125	Spr6p	125/125	No	No
3	144	Rim4p	143/144	1 base	1 amino acid
4	129	Spr6p	129/129	No	No
5	315	Bmh1p	312/315	3 bases	2 mutated bases at 3' non-translation region 1 base as Nonsense mutation
6	136	Rim4p	135/136	1 base	1 amino acid
7	205	Yrf1-7p	205/205	No	No
8	310	Bmh1p	307/310	3 bases	Nonsense mutation
9	207	Hxt5p	207/207	No	No
10	232	Agp3p	229/232	3 bases	3 amino acids
11	185	Mmt2p	182/185	1 base mutation and 2 bases deletion	Frameshift mutation
12	144	Spc19p	144/144	No	No
13	505	Rsa3p	504/505	1 base	Nonsense mutation
14	177	Spc19p	177/177	No	No

Bioinformatic analysis of differentially expressed genes

Among 14 differentially expressed gene fragments from yeast mutant strain N6076, 7 sequences revealed 100% homology with the original yeast strain sequence in the database; 6 gene fragments revealed 1 or 3 base mutations; in addition, Sequence 11 had 1 base mutation and 2 base deletions. Based on the gene prediction of amino acid sequences using software, the mutations of differentially expressed gene fragments and amino acids were shown in

Table 1. As shown in Figure 3, Sequences 3 and 6 revealed one amino acid mutation and Sequence 10 revealed 3 amino acid mutations. Through comparative analysis of Sequence 11 by BLASTN, the registered gene was Mmt2p with the deletion of 2 genes, thus resulting in frameshift mutation of the protein. On the other hand, through comparative analysis by BLASTX, there was no homologous sequence of Sequence 11 so that the functions of the protein were still unknown.

Fig. 3 Mutation status of amino acids in differentially expressed gene fragments. (a) mutation status of amino acids in Sequence 3, (b) mutation status of amino acids in Sequence 6, and (c) mutation status of amino acids in Sequence 10

DISCUSSION AND CONCLUSION

The original yeast strain was subjected to nitrogen ion irradiation and cell culture in shaking flasks as well as the examination of fermentation broths from mutant and original strains in thin layer chromatography. The yeast mutant strain N6076 could produce a new red substance due to the change of its metabolic pathways. The established subtractive library of mutant strain by SSH method revealed 14 gene sequences with various expression levels, which included 9 differentially expressed gene sequences. Among 14 differentially expressed gene fragments, 7 sequences revealed base mutation and 1 genes revealed base deletion. The functions of the protein based on amino acid prediction were still unknown after the comparison of homologous sequences. Similarly, on the basis of differentially expressed gene fragment analysis, nitrogen ion irradiation could result in the base mutation in the original strain.

Through analyzing the isolated differentially expressed genes involved in metabolic pathways, the yeast mutant strain N6076 after 96 h cultivation still revealed stronger metabolic capability than original strain. Among 7 isolated differentially expressed gene fragments with base mutation, 4 gene fragments revealed the change in protein sequences due to their base mutation. Whether the changed protein sequences can induce the functional change of the original proteins still needs to be further investigated through red substance involved in metabolic pathways.

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