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Genome Shuffling of *Penicillium citrinum* for Enhanced Production of Nuclease P1

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Abstract Genome shuffling is a powerful approach for efficiently engineering industrial microbial strains with interested phenotypes. Here we reported a high producer of nuclease P1, *Penicillium citrinum* G-16, that was bred by the classical physics-mutagenesis and genome shuffling process. The starting populations were generated by ^{60}Co γ-irradiation mutagenesis. The derived two protoplast fractions were inactivated by heat-treatment and ultraviolet radiation respectively, then mixed together and subjected to recursive protoplast fusion. Three recombinants, E-16, F-71, and G-16, were roughly obtained from six cycles of genome shuffling. The activity of nuclease P1 by recombinant G-16 was improved up to 1,980.22 U4/ml in a 5-l fermentor, which was 4.7-fold higher than that of the starting strain. The sporulation of recombinant G-16 was distinguished from the starting strain. Random amplified polymorphic DNA assay revealed genotypic differences between the shuffled strains and the wild type strain. The close similarity among the high producers suggested that the genetic basis of high-yield strains was achieved by genome shuffling.

Keywords *Penicillium citrinum* · Protoplast fusion · Nuclease P1 · Sporulation · Genome shuffling

Introduction

Nuclease P1 (E.C.3.1.30.1) is one member of zinc-dependent endonuclease family and produced by the mold *Penicillium citrinum* [1]. It contains three Zn(II) ions with two mononucleotide-binding sites [2]. The main function of this commercially available enzyme is to cleave single-stranded RNA and DNA into 5-mononucleotide, which is frequently used

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as an additive in food industry, an auxiliary therapy in hepatitis, nephritis, and muscle diseases [3], and agent in molecular biology research [4]. As a new biocatalyst for direct asymmetric aldol reaction under solvent-free conditions, the enzyme has been developed in biotransformation process [5].

To enhance the utilization efficiency of enzyme and production by *P. citrinum*, various processes have been investigated. DEAE cellulose [6] and marcoporous absorbent resin [7] as supports were evaluated for RNA hydrolysis in the continuous process. Gangadhara et al. [2] studied the effects of denaturants (GuHCl and urea) on nuclease P1 structure and activity, suggesting that the increased catalytic efficiency of nuclease P1 is due to a more open and flexible conformation of the activated enzyme. To the best of our knowledge, there were only a few reports on breeding of high nuclease P1 producers by *P. citrinum*. By the low-energy ion beam mutation treatment, strain N409 with the activity of nuclease P1 up to 421 U/ml was obtained [8]. Based on the means of mutations with ^{60}Co γ-irradiation and nitrosoguanidine (NTG), the high-yield mutant HEP2312 (nuclease P1 at 1,508 U/ml), was screened [9]. Genome shuffling is a powerful approach for evolution of industrial strains with desirable phenotypes [10–12]. Recently, genome shuffling has been used to improve production of the low-temperature alkalophilic lipase by *Acinetobacter johnsonii* [13], xylanase activity by *Apsergillus* sp. NRCF5 [14], ε-poly-L-lysine production by *Streptomyces graminearus* [15], and 1,3-propanediol by *Clostridium diolis* DSM 15410 [16]. These reports demonstrated that genome shuffling for phenotypic improvement was efficient and effective [17].

The aim of this study is to breed high-yield producers of nuclease P1 by induction of mutations and genome shuffling. The protocols for isolating, regenerating protoplasts, and successive rounds of protoplast fusion in *P. citrinum* were described. A high producer of nuclease P1 (recombinant G-16) was obtained, and its productivity was confirmed in 5 liter fermentor.

Materials and Methods

Fungal Strains and Culture Conditions

Wild type of *P. citrinum* CCICC 4011 was obtained from China Center of Industrial Culture Collection (Peking, China), can produce nuclease P1 activity at 226 U/ml. The fungus was maintained on potato dextrose agar (PDA) medium at 28 °C for 3–5 days when spores were formed [8]. For nuclease P1 production, glucose and peptone (GP) medium (glucose 70 g, peptone 5 g, $K_2HPO_4 \cdot 3H_2O$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.4 g, $ZnSO_4 \cdot 7H_2O$ 0.4 g, $CaCl_2$ 0.4 g, pH 6.5, per liter deionized water) was prepared.

Nuclease P1 production process in a 5-l fermentor: a loop of PDA culture was inoculated into 100 ml of GP medium in a 500-ml Erlenmeyer flask. After incubation at 28 °C, 200 rpm for 30 h, 100 ml seed broth was transferred into a 3.5-l GP in a 5-l stirred-tank fermentor (B. Braun Inc., German) and incubated at 28 °C, 300 rpm, and 1.0 vvm aeration rate.

Preparation of Starting Strains for Genome Shuffling

The starting strains for genome shuffling were obtained through subtle improvements of the wild-type *P. citrinum* 4011 by ^{60}Co γ-irradiation with 300 Gy dosage treatment [9, 18]. Briefly, 5 ml of spore suspension from a slant culture were transferred to an aseptic tube. ^{60}Co γ-irradiation was used for mutation treatment. After appropriately diluting, the suspension of spores was spread on the PDA medium. A number of colonies were selected, and

their nuclease P1 activity was evaluated by fermentation tests. The mutants with higher production were preserved and used as the starting strains for genome shuffling.

Protoplast Preparation and Inactivation Treatment

Protoplasts were prepared according to a previously reported method by a few modification [19]. Briefly, the uniform conidial suspensions of selected strains were inoculated in 300 ml Erlenmeyer flasks containing 30 ml PDA medium. The flasks were cultivated on a rotary shaker 100 rpm overnight at 28 °C. Mycelium was collected by filtratin through sterile Myracloth, rinsed with and resuspended in 0.6 M NaCl.

DL-dithiothreitol (DTT) solution was used to pretreat the pellets in 100 ml flask on a rotary shaker (100 rpm) for 30 min. Rinsed with 0.6 M NaCl to discard DTT, 250 mg (wet weight) of the pellets was resuspended in 10 ml of the solution of cell wall-lytic enzymes (cellulase, snailase, and lyticase, 2 mg/ml), cultured at 30 °C on a shaker incubator operating at 80 rpm in 50 ml Erlenmeyer flask until maximum protoplastation. Protoplasts were separated from the mixtures by filtration through sterile Myracloth, and the number of protoplasts in the filtered solutions was counted under a microscope using hemacytometer. After centrifuged at 800×*g* for 15 min in 0.6 M NaCl, protoplasts were washed and recovered.

To improve the efficiency of hybrid screening, parental starting protoplasts were applied to inactivation treatment, then the parental protoplasts cannot grow on regeneration plates. The equal number of protoplasts from starting populations was mixed and then divided equally into two aliquots [15]. One aliquot was inactivated by heat at 50 °C for 30 min, the other was treated by UV inactivation under a 30 W UV lamp for 15 min.

Genome Shuffling

Protoplasts were fused using a modification of the method of Lv et al. [18]. Aliquots of inactivated protoplasts were mixed in a ratio of 1:1, collected by centrifugation and then resuspended in PN solution (40 % PEG-6000 in 0.6 M NaCl buffer, pH 7.8). The mixture was incubated at 30 °C, 80 rpm for 30 min. The pellets were centrifuged, washed twice with 0.6 M NaCl buffer (pH 7.8), and resuspended in 0.6 M NaCl buffer (pH 7.8). It was serially diluted and regenerated on regeneration medium (PDA medium supplemented with 0.6 M NaCl as osmotic stabilizer) plates for 3–7 d at 28 °C. The colonies were isolated and the productivity was evaluated with fermentation test in shaking flasks. The selected high-yield fusants were used as the starting strains for subsequent rounds of genome shuffling. Six successive rounds of genome shuffling were performed. Stable segregants, suggested to be haploid, were obtained by growing the hybrids on PDA containing benlate at a concentration of 0.5 μg/ml. The segregants were checked for stability on fresh medium containing benlate and subsequently phenotypically characterized by replication onto appropriately PDA media [20].

Random Amplification Polymorphic DNA Analysis

Genomic DNA extraction and random amplified polymorphic DNA (RAPD) analysis were performed as described by Abe et al. [18, 21, 22], respectively. DNA was extracted from mycelia of *P. citrinum*. The mycelia were grinded to be powder under liquid nitrogen condition and suspended in liquid (0.1 M NaCl, 30 mM Tris–HCl pH 7.8, 10 mM EDTA). For the RAPD analysis, the primers shown in Table 1 were used. Amplification was achieved using a thermal cycler set at the following program:

Table 1 Primers and their nucleotide sequence used for RAPD s199, s197, s156, s155, s104, and s10

Primer	Sequence
S199	GAGTCAGCAG
S197	TGGGGACCAC
S156	GGTGACTGTG
S155	ACGCACAACC
S104	GGAAGTCGCC
S10	CTGCTGGGAC

94 °C for 4 min, followed by 40 cycles of 94 °C for 30 s, 34 °C for 1 min, and 72 °C for 2 min and extended by 72 °C for 5 min, with the reaction system: 5 μl 10×PCR buffer, 3 μl $MgCl_2$ (25 mM), 1 μl dNTP(10 mM), 2 μl primers, 2 μl DNA template, 1 μl Taq polymerase, and 36 μl sterile water. The products were separated by electrophoresis in 2.0 % agarose gels. The presence of amplified bands with different intensities and locations were detected and analyzed with the Quantity One 4.1 (BioRad, Hercules, CA, USA) software. The RAPD band patterns were analyzed by the unweighted pair group method using arithmetic average (UPGMA) method.

Nuclease P1 Assay

The activity of nuclease P1 was determined by the method of Ying et al. [23]. In brief, 1.9 ml substrate (containing 1 % RNA (*w*/*v*), 125 mM acetic acid buffer (pH 5.1), 1 mM Zn(II)) was heated at 67 °C in the constant temperature water-bath and keep warm, then 0.1 ml diluted enzyme was added and incubated for 15 min. The test tubes were transferred into ice water, 2.0 ml precooling of nucleic acid precipitant (0.25 % $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$–2.5 % $HClO_4$ (*w*/*v*)) was added to stop the reaction, and kept in the ice bath for 20 min. The precipitated RNA was removed by centrifugation. The absorbance at 260 nm of the supernatant was recorded. The enzyme activity (U) was calculated as the below:

$$\text{Enzyme activity } \left(\text{U}\Big/\text{ml}\right) = \frac{\Delta\text{O.D.} \times 4 \times 50}{0.1 \times 15} = \Delta\text{O.D.} \times \text{a} \times 133.3$$

Where *a* is the dilution times of the enzyme before enzyme activity determination.

Statistical Analysis

All the data were subjected to statistical analysis by one-way ANOVA at $p<0.05$ and $p<0.01$.

Results

Strains Mutagenesis and Starting Strains Selection

The feature of natural evolution is practically mimicked by genome shuffling on recursive genetic recombination. The starting population with an improvement of the desired phenotype from the wild-type is required before genome shuffling. ^{60}Co γ-irradiation was used as

a mutagenic agent to improve the enzyme productivity of the wild-type strain. Mutant selection was based on the evaluation of flask tests. Six mutants with an improved activity, named as CM-11, CM-42, CM-33, CM-97, CM-158, and CM-227, were selected from a mutant library of 306 colonies respectively (Fig. 1). In the flask tests, the nuclease activity by mutant CM-11 was up to 420.22 U/ml and 0.86-fold increase over *P. citrinum* 4011 (nuclease activity 226 U/ml). Consequently, CM-11, CM-42, CM-33, CM-97, CM-158, and CM-227 were used as the starting population for genome shuffling.

Optimization of Protoplast Preparation and Regeneration Conditions

To conduct the protoplast fusion, various factors of protoplast formation and regeneration were investigated (Table 2). Mycelia pretreated with DTT for 45 min was helpful to the rate of protoplast formation by 54 %. Four osmotic stabilizers were tested for their efficacy in releasing protoplasts. When 0.6 M NaCl was used as osmotic stabilizer, the maximum numbers of protoplasts were obtained. For the release of protoplasts, three commercially available enzymes were investigated for their lytic activity against *P. citrinum* mycelia. Enzyme mixture consisting of cellulose/snailase/-lyticase (in a ratio 2:1:1 for 140 min) were able to produce the maximum rate of protoplasts formation (Table 2 and Fig. 2). The process of protoplast formation from mycelia was clearly shown in Fig. 3.

Genome Shuffling of Improved Mutant Populations

Genome shuffling is depended on the high frequency of protoplast formation and fusion, while the recursive fusion of protoplasts that permits obtaining quickly the phenotype of interest is the basis of genome shuffling [24, 25]. To bleach parental populations from

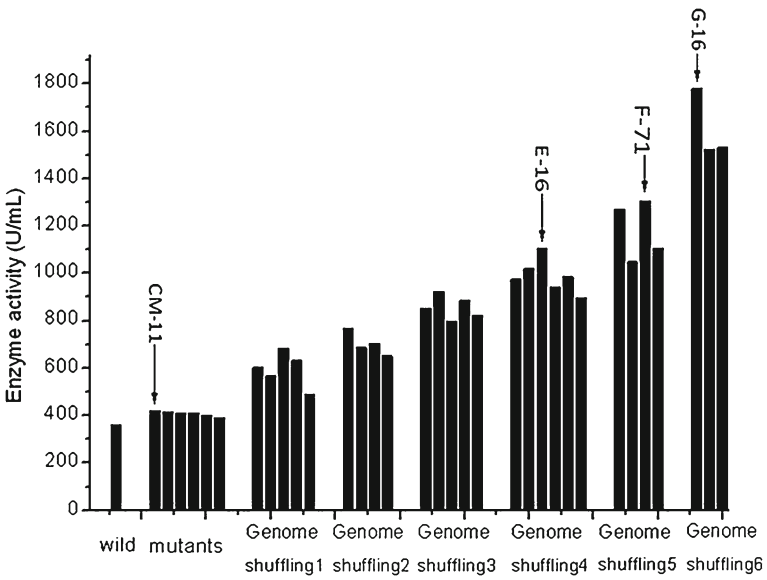

Fig. 1 Improvement of nuclease P1 activity by genome shuffling. One to six rounds of genome shuffling were used to improve nuclease P1 activity of *P. citrinum*

Table 2 Effects of different treatment on rate of protoplast formation

Factors	Time of DTT[a]					Osmotic stabilizer[b]						Proportion of enzymes mixture[c]			
	0	15	30	45	60	NaCl	KCl	Sorbitol	Mannitol	Sucrose	$MgSO_4$	1:1:1	2:1:1	1:2:1	1:1:2
Rate of protoplast formation[d]	24	33	50	54	53	47	8.7	0.9	0.7	0.9	5.0	47	69	52	45

[a] The times of DTT treatments are 0, 15 30, 45, and 60 min, respectively

[b] The concentration of all kinds of osmotic stabilizer is 0.6 mol/l

[c] The proportion represents the volumes of the adding enzymes with a same concentration

[d] Rate of protoplast formation (%)

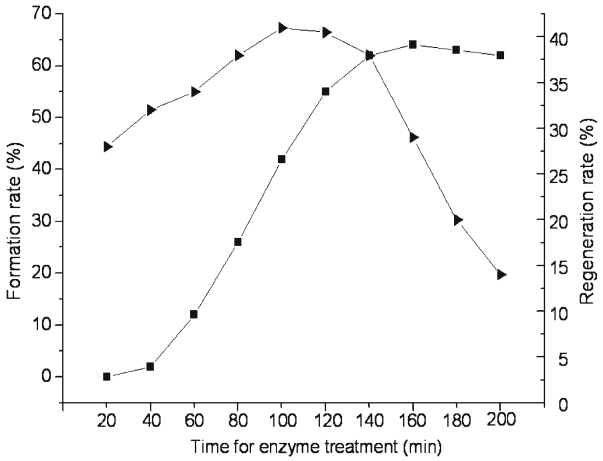

Fig. 2 The optimum time of enzyme treatment. With the process of enzyme treatment, protoplast formation rate (*filled square*) continuously increased until 160 min, while protoplast regeneration rate (*filled triangle*) was first rising, reached the maximum at 100 min, and then decreased quickly

fusants, UV radiation and heat treatment were utilized [26]. After UV radiation for 15 min or heating at 55 °C for 30 min, the inactivation rate of protoplasts of *P. citrinum* was up to 100 % (Table 3). The temperature and time in the fusion process were further evaluated too. Good fusion rate would be produced at 30 °C for 15 min (Fig. 4).

Genome shuffling was applied to improve nuclease P1 production by the six mutants, CM-11, CM-42, CM-33, CM-97, CM-158, and CM-227, obtained by ^{60}Co mutagenesis. After the first round of shuffling, 200 colonies were selected. Through the enzyme activity assay by flask tests, five recombinants, G1-7, G1-19, G1-163, G1-192, and G1-195, showed high enzyme activities and were pooled as starting populations for the next shuffling process. After six successive rounds of genome shuffling, from the 1183 shuffling colonies three recombinants (E-16, F-71, and G-16) were selected by their nuclease P1 activity (Fig. 1). The enzyme activity by recombinant G-16 was up to 1762.49 U/ml, 4.2 times higher than that of the starting strain CM-11 (420.22 U/ml). The genetic stability of G-16 was evaluated by five successive subcultivation tests on PDA containing benlate at a concentration of 0.5 μg/ml. The rang of production levels among five generations was

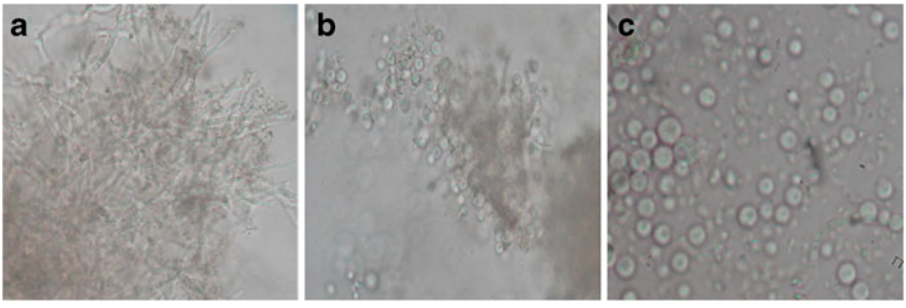

Fig. 3 The process of protoplast formation. **a** Mycelia grew rapidly in the initial stage. **b** Protoplast formation under the optimal conditions of protoplast formation and regeneration. **c** A great number of protoplasts were obtained

Table 3 Effects of various factors on inactivation of protoplasts for fusion

Factors	Death rate (%)	Regeneration rate (%)
UV inactivation[a]		
5	87.5	2.1
10	93.3	1.4
15	100	0
20	100	0
25	100	0
Heat inactivation[b]		
45	68	6.7
50	94	1.3
55	100	0
60	100	0
65	100	0

[a]The times of UV inactivation are 5, 10, 15, 20, and 25 min, respectively[b]The temperatures of heat inactivation are 45 °C, 50 °C, 55 °C, 60 °C, and 65 °C, respectively

1,684.3 to 1,792.7 U/ml, indicating that the hereditary characters of the high nuclease P1 producing recombinant, *P. citrinum* G-16, was stable.

Morphological Characteristics of Parental and the Shuffled Strains

The size of mycelium was measured at the beginning of the cultivation on PDA agar. Figure 5 showed the comparison of the morphological characters of mycelia, pycnidia, and conidiospores development between the recombinant G-16 with the parental strain 4011. Cultured on PDA agar surface, the recombinant G-16 achieved a quick

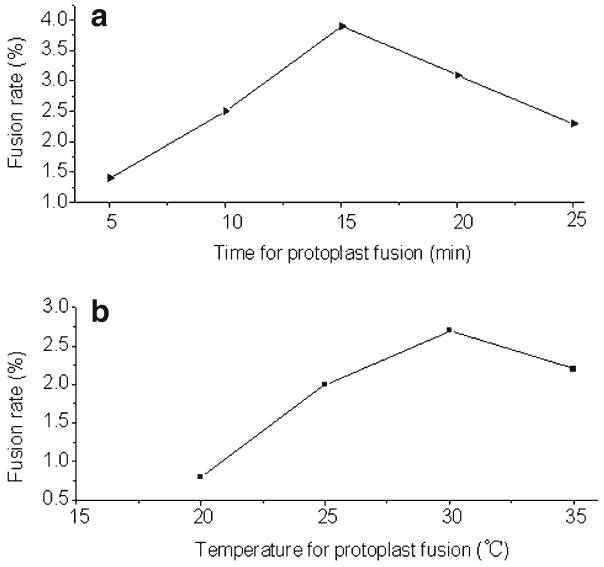

Fig. 4 The conditions required for protoplast fusion process. **a** Time effect on the fusion rate. **b** Temperature effect on the fusion rate

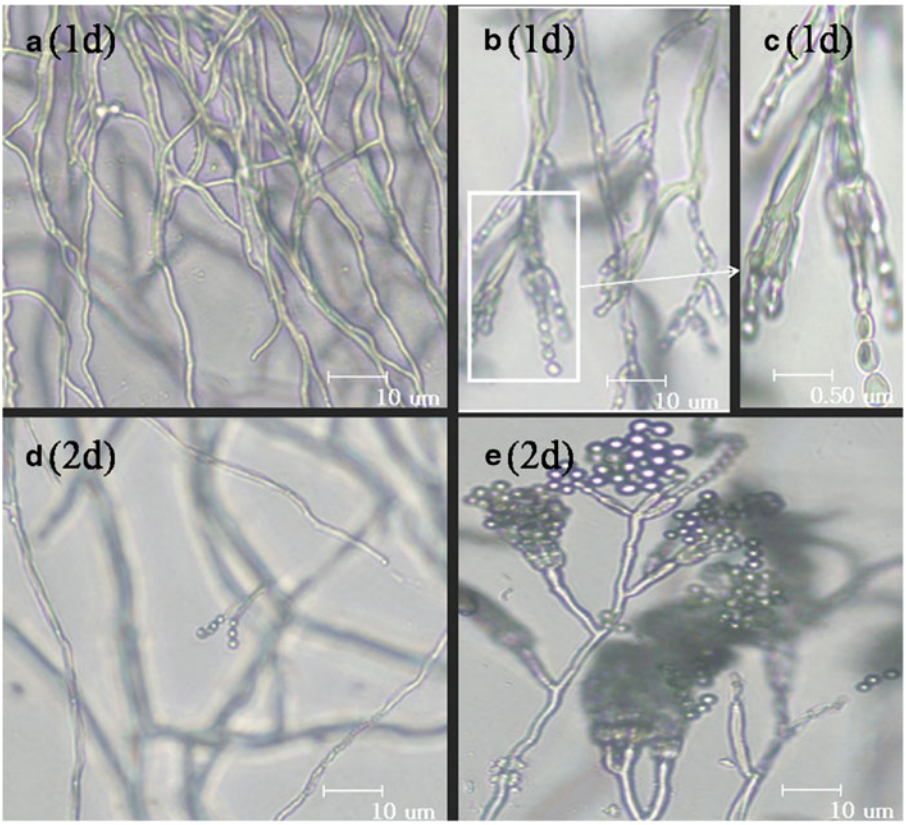

Fig. 5 Comparison of sporulation by the recombinant G-16 and the parental 4011. **a**, **d** The mycelia and little conidiospore of parental 4011 within 1 and 2 days growth, respectively. **b**, **c**, **e** Tthe abundant conidiospores produced by the recombinant G-16 within 1 and 2 days, respectively

development process of mycelium into sporulation and produced a great deal of conidiospores (within 2 days, 28 °C). However, strain 4011 exhibited good growth of mycelia and little formation of conidiospore. This result suggested that improved spore productivity may be correlated with metabolite synthesis, such as nuclease P1.

Characterization of Nuclease P1 Production Profiles of the Shuffled Strain

Nuclease P1 production and cell growth of the recombinant G-16 was further examined in a 5 liter fermentor (Fig. 6). Consistent with the results obtained in flask tests, recombinant G-16 produced more than 4.7-fold nuclease P1 yield (1,980.22 U/ml), compared with that of the starting strain after 84 h. As shown in Fig. 6 nuclease P1 was produced along with exponential growth in the fermentation process. Sugar was consumed rapidly in the exponential growth phase. The mycelium growth was almost consistent with the sugar consumption and stop in the stationary phase. These results implied that there were two rapid growth phases, M1 (12–36 h) and M2 (48–72 h). Corresponded to M1 transition into M2 phases, the producing rate of nuclease P1 was changed to a higher level, which was consistent with the sporulation time (Fig. 5). Although mycelium biomass growth stop at 72 h, nuclease P1

production continued and reached the peak at 84 h (Fig. 6). The amino nitrogen was consumed fast in the first 24 h of cultivation, which corresponded with sugar consumption and pH decreased. After that, amino nitrogen concentration increased sharply with 50 h and then decreased slowly again.

Polymorphism of Genomic Population by RAPD Analysis

RAPD analysis was used to examine the genetic diversity among 6 shuffled recombinants with different enzyme productivity and the parental strain 4011. Six of the pre-screened primers (s199, s197, s10, s155, s104, and s156) successfully amplified polymorphic DNA bands. Amplified bands were characterized based on their size, which ranged from approximately 150 to 2,500 kb. RAPD band pattern was analyzed using the Nei similarity index, and a dendrogram was constructed based on the similarity matrix data by applying the UPGMA cluster analysis method (Fig. 7). The shuffled recombinants were segregated and high producers (E-16, F-71, and G-16) were clustered into a subgroup. Based on the dendrogram, close homology existed in those strains that the similarity coefficient of the seven strains varied from 0.58 to 0.77. The shuffled recombinant G-16 was the closest to F-71 (their genetic similarity coefficient was 0.77, while genetic distance was 0.185), but was distant to the low producer E-9 (their genetic similarity coefficient was 0.58, while genetic distance was 0.462). The specific bands and UPGMA dendrogram of cluster analysis obtained in our study showed that genetic information was transferred from the parental strains to the shuffled strains by genome shuffling and the genetic information of the shuffled strains had been changed.

Discussion

Nuclease P1 is widely produced by various industrial process. Exploring more effective method to increase the enzyme production is the interest for its manufacture. In the present

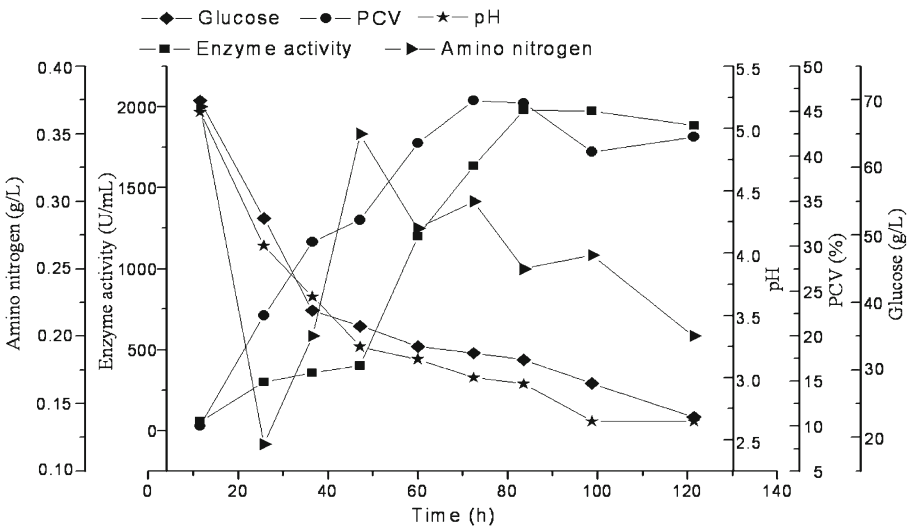

Fig. 6 Time course of nuclease P1 production by recombinant G-16. PCV (Packed-cell volume) glucose, pH, enzyme activity (nuclease P1), and amino nitrogen were assayed

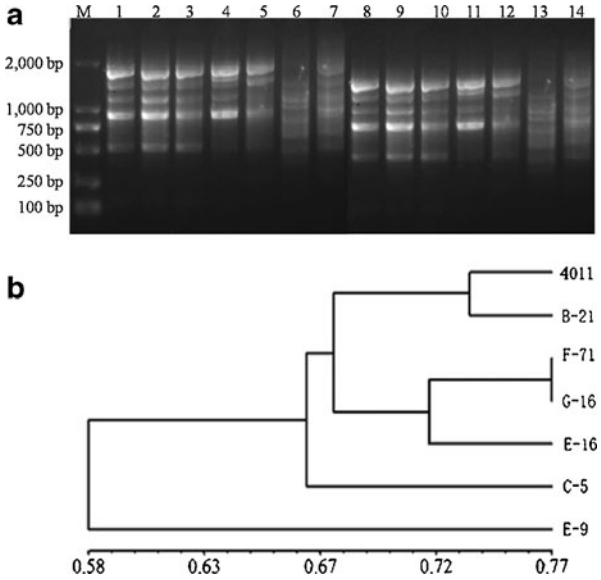

Fig. 7 Polymorphism of genomic population by RAPD analysis. **a** RAPD-PCR profiles of strains using primer s199 and s156. *M* is the DNA marker. *Lanes 1–7* are the parent strain and the high-yielding isolates (from *left* to *right*: 4011, F-71, B-21, G-16, C-5, E-16, E-9) by using primer s199, while *lanes 8–14* are the parent strain and the high-yielding isolates (from *left* to *right*: 4011, F-71, B-21, G-16, C-5, E-16, E-9) by using primer s156. **b** Dendrogram of the high/low nuclease P1-producing mutants based on UPGMA cluster analysis and similarity index

study, we successfully applied genome shuffling method to improve production of nuclease P1. After six successive rounds of genome shuffling, the recombinant G-16 with high production was picked up from 1183 shuffled isolates. The enzyme activity by recombinant G-16 was up to 1,762.49 U/ml, 4.2-fold higher than the starting strain CM-11 (420.22 U/ml; Fig. 1). This improvement was at similar level as that of previous report for *S. fradiae* (6-fold) [11], *Lactobacillus* (3-fold) [25], and *Streptomyces* sp. U121 (5-fold) [27]. In the 5-l fermentor, the enzyme production was increased further to 1,980.22 U/ml (Fig. 6). The production of G-16 was higher compared to other strain development efforts that have been reported [8, 9]. For example, Li et al. (2007) screened a nuclease P1 producer of *P. citrinum* by ultraviolet mutagenesis, its activity reached 1,508 U/ml [9]. Therefore, recombinant G-16 has a potential application in the industry.

One clue of the improved performance of recombinant G-16 was its differentiations in morphological and genetic characteristics compared to the starting strains (Figs. 5 and 7). Recombinant G-16 had a high productivity of conidiospores in 48 h growth, while the parental strain 4011 was sparse. It has been reported that the ability of producing enzyme is related directly to the ability of sporulation by microbe [28]. In *Aspergillus niger*, a predictable behavior fungal morphology was correlated closely with productivity [29]. In *S. coelicolor*, some associations between the sporulation and secondary synthesis were often observed [30]. Therefore, the colonies with abundant conidiospores would be hyper-producers of the enzyme.

Molecular genetic techniques, such as amplified fragment length polymorphism (AFLP) [31] or RAPD [21, 32], have become powerful tools to analyze genetic

relationships and diversity. In this study, polymorphism of DNA RAPD was utilized to investigate the genetic variation and evolution between fusants and parental strains. As shown in Fig. 7, the higher producers were clustered together (E-16, F-71, and G-16) and were separated from those low-yield strains. Taken together with the results of morphological and metabolic characteristics, our results implied the production improvements of fusants are derived from genetic variation, not from modification. Hence we hypothesized that some unique DNA fragments correlated with hyper-yielding characteristics was common among the hyper-yielding shuffled strains.

Conclusion

In this study, we described a genome shuffling protocol for isolating, regenerating protoplasts, and successive rounds of protoplast fusion in *P. citrinum*. Our results demonstrated that rapid improvement of nuclease P1 activity was achieved by this method. The starting mutant population was generated by ^{60}Co γ-irradiation treatments of the spores. After six rounds of protoplast fusion, an improved recombinant G-16 was obtained and its activity yield of nuclease P1 reached 1980.22 U/ml in a 5-l fermentor. The morphological and molecular difference between the parent and shuffled recombinants were observed, and the mechanism of the improved shuffled recombinants was discussed. Our results suggested that G-16 would be a promise candidate strain for industrial application, and genome shuffling is a powerful approach for molecular breeding of industrial strains.

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Conflict of interests The authors declare that they have no conflict of interests.

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