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Full Length Research Paper

Influence of mixed culture system on the growth performance of *Aspergillus oryzae* and *Saccharomyces cerevisiae*

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This study describes a novel strategy to improve the α-galactosidase and invertase production of *Saccharomyces cerevisiae* by co-cultivating it with *Aspergillus oryzae*. In the mixed culture, the growth of the both strains was repressed, and the protein synthesis for the yeast cell wall was promoted significantly. As a result, α-galactosidase and invertase activities of the mixed culture reached 85.16 and 561.60 Uml^{-1}, over 9 and 15 fold greater than the values obtained in the cultures of single strain, respectively. During the simultaneous saccharification and fermentation procedure, ethanol with the concentration of 15.2% (v/v) was obtained from soybean sirup in 60 h at 30°C, and the conversion efficiency of total sugar to ethanol was 96.3% of the theoretical ethanol yield.

Key words: Co-cultivation, *Aspergillus oryzae*, *Saccharomyces cerevisiae*, α-galactosidase, invertase.

INTRODUCTION

Global production of soybean for 2006 was estimated at 227 milliard tons, and soybean meal was the main solid by-product in soybean industry (Keith, 2007). However, soybean meal was composed of high concentration of raffinose and stachyose which were not digestible in the intestines of monogastric animals (Liu et al., 2006). α-Galactosidase (α-Gal. E.C. 3.2.1.22), which is distinguishable from invertase (β - D - fructan fructohydrolae, E.C. 3.2.1.80) by its ability to hydrolyze melibiose, catalyzes the hydrolysis of raffinose and stachyose by splitting off D-galactose units by cleaving the glycosidic bonds of α (1→6) (Kotiguda et al., 2007). Thus, microbial α-galactosidase and invertase play an important role in the hydrolysis of soybean meal for its commercial exploitation.

A number of bacterial, yeast, and fungal strains produce α-galactosidase and invertase. Among them, fungal and yeast strain is the most common and preferred choice (Liu et al., 2006; Kotiguda et al., 2007). However, there are few data for production α-galactosidase in submerged system with the fungal strains, as most researchers have worked with solid-state cultivation system which was not favorable to be scaled-up for industrialization (Kotiguda et al., 2007; Szendefy et al., 2006). This may be due to low performance of these strains in submerged system where the enzyme synthesis was inhibited by catabolite repression (Maldonado et al., 1989). Much work has been done to enhance the activities of α-galactosidase from the yeast, such as cell immobilization technique, identifying the genes which code for the α-galactosidase and so on. It was previously found that α-galactosidase and invertase synthesis was inducible (Shankar and Mulimani, 2007; Zhang and Ge, 2006), therefore, to enhance the α-galactosidase and invertase synthesis of yeast in submerged system, it was essential to decrease the total monosaccharides concentration in the medium, meanwhile redistribute the *in vivo* carbon and energy flux for enzymes synthesis (Ge et al., 2008).

In this article, *A. oryzae* L-09 was inoculated to the medium of .*S. cerevisiae* Z - 06 to exhausted the monosaccharides in the submerged system, leading to the

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induction of α-galactosidase and invertase. Also, high concentration ethanol was obtained from the soybean with favorable conversion efficiency. The inducing and stressing mechanism for the enzymes synthesis in the mixed culture are discussed.

MATERIALS AND METHODS

S. cerevisiae Z-06 used throughout this study was origi-nally isolated from a fermented mash (Ge and Zhang, 2005), *A. oryzae* L-09 were isolated from the mildewy corn and identified as described by Dai (1987). The two strains were grown on malt and potato agar slants at 30°C for three days and then stored at 4°C until use (Shen, 1999).

The raw soybean sirup used in this study was presented by ShanDong ChangRun Biology Co. LTD, and contained 34.3% (w/w) of total reducing sugars. The wheat brans and soybean flour were acquired from local suppliers, and passed through an 80 mesh sieve, other chemicals were certified reagent grade.

Cultivation

The medium for production of α-galactosidase and invertase contained (per L) 100 g of raw soybean sirup, 30 g of soybean flour, and 30 g of wheat brans, pH 5.4. The above medium was cooked at 100°C for 1 h, and filtrated by eight-folds gauze to remove the insoluble materials. To prepare the inoculums, a 2 ml of suspended yeast cell (with cell count of 10^8 ml^{-1}) from the slant was transferred into a 250 ml Erlenmeyer flask containing 50 ml medium. The shake flasks were incubated at 30°C for 48 h on a rotary shaker (140 rpm) and then inoculated into a 7 L jar bioreactor (KF - 7 L; Korea Bioreactor Co., Inchon, South Korea) with a working volume of 4 L. The aeration rate was 1.125 vvm, and the agitation speed was automatically controlled at a set point of 140 rpm.

Production of α-galactosidase and invertase in the mixed culture of *S. cerevisiae* and *A. oryzae*

It was previously found that the production of gluco-amylase can be ceased by the glucose in the culture (Rajoka and Yasmeen, 2005). Therefore, to further enhance the enzyme activities, a 2 ml of *A. oryzae* L-09 spores suspension (10^6 to 10^7 spores/ml) was inoculated to the culture to exhaust the monosaccharides of the medium. The culture without the inoculation of *A. oryzae* was used as a control.

In order to investigate whether the inoculation of *A. oryzae* to the medium was essential for the enhanced enzyme activities, the fungi was inoculated to the basal medium at different times (at the beginning, and in 6 h intervals).

Enzymes analysis and the preparation of cell extract

The submerged culture of *S. cerevisiae* Z-06 and *A. oryzae* L-09 were grown aerobically for 48 h, and then filtrated by 8-folds gauze to separate the fungal pellets from the yeast cells. The results of the separation were checked using microscopic observations. The filtrated culture was diluted and used to assay the enzyme activities without centrifugation. To harvest the yeast cells, the above filtrated culture was centrifuged (5,000 × *g* for 5 min at 4°C). The yeast cells were washed twice with ice-cold saline (0.85% NaCl wt/vol) and resuspended in 25 ml of 200 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA. The cells were disrupted ultra-sonically at 4°C for 300 cycles of 4 s (ACX 400 sonicator at 20 kHz; Sonic and Materials, Newton and Mass). Cell debris (CD) was obtained by centrifugation (10,000 × *g* for 5 min at 4°C), which also resulting in a cell extract (CE). Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard. *S. cerevisiae* CD was resuspended in precooled 100 mM McIlvaine buffer (pH 5.0) for protein and enzymes analysis.

Influences of the mixed culture on the morphological and physiological characteristics of *S. cerevisiae* Z-06 and *A. oryzae* L-09

The mycelia pellets of *A. oryzae* L-09, as well as the cells of *S. cerevisiae* Z-06 from the mixed culture were washed twice with ice-cold saline (0.85% NaCl, wt/vol). The mycelia pellets were resuspended in 200 mM potassium phosphate buffer (pH 7.0) and observed directly. The yeast cells were dyed by crystal violet, safranin or other dyes (10 g l^{-1}) for 0.5 min, washed immediately by distilled water, then examined using biological micro-scope (XSZ-G), and that from the culture of the single strain was used as a control.

Ethanol fermentation from raw corn flour by simultaneous saccharification and fermentation procedure

The saccharification and fermentation of soybean sirup proceeded simultaneously within the 3 l fermentor containing 1.5 l liquid mixed culture of *A. niger* SL-09 and *S. cerevisiae* Z-06, and the medium was stepwise supplemented with 1.5 l of soybean sirup then incubated statically at 30°C. During the fermentation process, liquid culture was periodically withdrawn and used for the analysis of starch, ethanol, total sugar and reducing sugar.

Analytical methods

The α-galactosidase activity was assayed in test tubers as described by Garro et al. (2004). The reaction mixture contained: 10 mM p-nitrophenyl-β-d-galactopyranoside (pNPG) 50 µl, 100 mM McIlVaine buffer pH 5.0 50 µl, enzyme sample 100 µl, final volume 200 µl. The mixture was incubated at 50°C for 10 min, and the reaction was stopped by adding 3 ml of sodium carbonate (0.25 mM). One unit (U) of α-galactosidase activity was defined as the amount of enzyme that liberates 1 µmol of pNP from pNPG per min. The invertase activity was assayed as described by Pessoni et al. (1999). One unit (U) of invertase activity was defined as the amount of enzyme that release 1 µmol of fructose from sucrose per min. Reducing sugars were estimated with 3,5-dinitrosalicylic acid (DNS), using glucose as standard. Total reducing sugar was assayed by the same method after acid hydrolysis (pH adjusted to 1 with H_2SO_4; 30 min at 100°C). Yeast cell populations were determined by direct micro-scopic count in a counting chamber.

Biomass concentration of the fungi was determined by harvesting the mycelia pellets by filtration and freeze-drying them to a constant weight, while the dry weight was expressed as gram per litre of the culture. To measure the concentrations of glucose, fructose, galactose, sucrose, melibiose, raffi-nose, and stachyose of the culture, the samples were centrifuged (10,000 × *g* for 10 min at 4°C), extracted by 75% (vol/vol) ethanol, then measured by high performance liquid chromatography (HPLC, Waters Associates model 209, equipped with a differential refractive index R1401 detector; using Merck Lichrosorb-NH_2 column (0.46 × 25 cm), at 30°C. A mixture of acetonitrile-water (60:40 vol/vol) was used as the mobile phase at a flow rate of 1.0 ml min^{-1}). All the experiments were repeated three times, and the values are expressed as the means of duplicate measurements on three independent samples. The

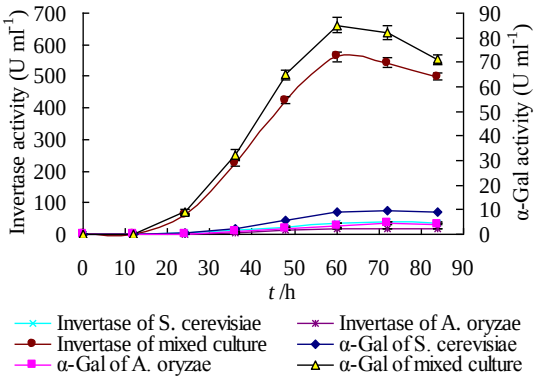

Figure 1. Time course of α-galactosidase and invertase production in the control and the mixed culture of *S. cerevisiae* Z-06 and *A. orgzae* L-09, and the *A. oryzae* L-09 was inoculated at the beginning of the mixed culture.

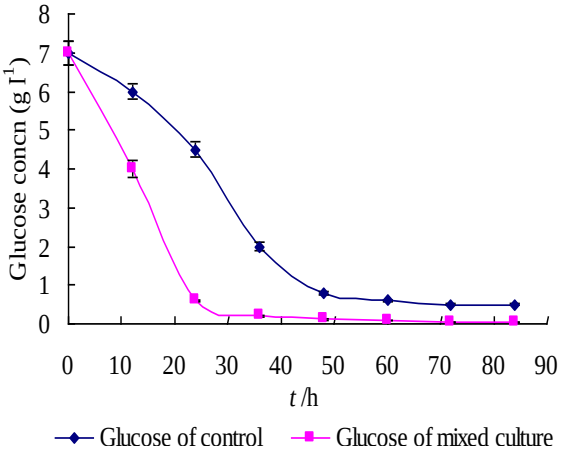

Figure 2. Glucose concentration during the culture of control and the mixed culture, and the *A. oryzae* L-09 was inoculated at the beginning of the mixed culture.

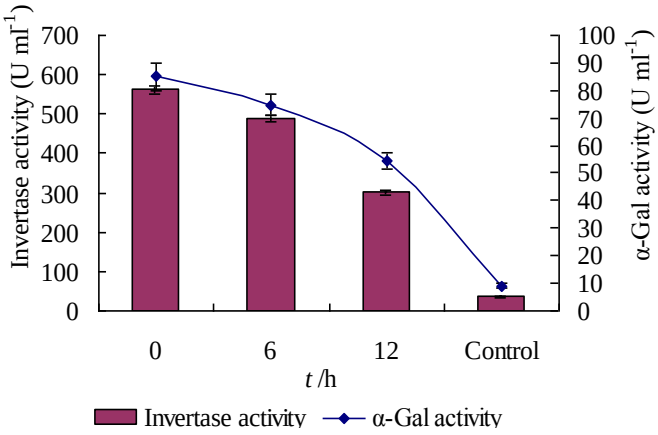

Figure 3. Enzyme production of in the mixed culture of *S. cerevisiae* Z-06 and A. orgzae L-09. The A. oryzae was inoculated to the culture at different times (at the beginning, and in 6 h intervals), and the culture without inoculation used as a control.

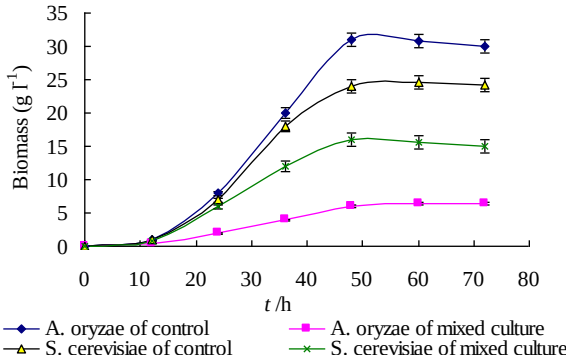

Figure 4. Biomass of *S. cerevisiae* Z-06 and *A. oryzae* L-09 in the control and mixed culture, and the *A. oryzae* L-09 was inoculated at the beginning of the mixed culture.

data were analyzed by SAS software (USA).

RESULTS

Enzyme production of *S. cerevisiae* Z-06 in the mixed culture

As shown in Figure 1, maximum α-galactosidase and invertase activities of 85.16 and 561.60 U ml^{-1} were obtained in the mixed culture, which were over 9-fold and 15-fold greater than the values obtained in the control (9.65 U ml^{-1} for α-galactosidase, and 38.64 U ml^{-1} for invertase), respectively; and the culture time was shortened from 72 to 60 h. To determine the effect of *A. oryzae* inoculation on the medium composition, the glucose concentration of the control and mixed cultures were measured. As shown in Figure 2, the inoculation of *A. oryzae* decreased the glucose concentration significantly, which was lower than 1 g l^{-1} from 24 h of the culture. Therefore, the low performances in the control may attribute to the high concentration of glucose, which can repress the enzymes synthesis dramatically (Maldonado et al., 1989).

Figure 3 presented the enzymes production of *S. cerevisiae* Z-06 with the inoculation of *A. oryzae* L-09 at each stage of the culture. The enzyme activities obtained were all higher than that of the control, and only in case of fungal-supplementation at the beginning 6 h of the culture, more than 70 and 400 U ml^{-1} for α-galactosidase and invertase activities were obtained, respectively.

Effect of the mixed culture on the growth performance of the both strains

As shown Figure 4, in the mixed culture, the biomasses of both strains were decreased dramatically. The biomass of the *A. oryzae* L-09 in the mixed culture was only 20%

Table 1. Enzymes activities and protein concentration of the control and mixed culture[a].

Parameter	Single *S. cerevisiae* Z-06			Mixed culture		
	P[b] (mg ml^{-1})	A[c] (U ml^{-1})	I[d] (U ml^{-1})	P[b] (mg ml^{-1})	A[c] (U ml^{-1})	I[d] (U ml^{-1})
Culture Supernatant	1.12±0.05	0.54±0.05	2.26±0.09	1.24±0.06	3.63±0.08	16.84±0.43
Suspension cells	20.15±0.55	8.63±0.15	36.15±0.95	26.61±0.85	83.91±0.90	561.56±1.55
Cell extract	10.16±0.46	4.11±0.15	18.34±0.80	10.54±0.59	5.77±0.38	20.61±0.86
Resuspended cell debris	20.23±0.40	7.96±0.10	33.63±0.55	28.53±0.88	80.51±0.54	558.88±1.89
Total value	31.51±0.91	12.61±0.30	54.23±1.44	40.28±1.53	89.91±1.0	596.33±3.18

[a] The submerged culture of the control and the mixed culture of *S. cerevisiae* Z-06 and *A. oryzae* L-09 were grown aerobically for 60 h. The yeast cells obtained from the control and the mixed culture were resuspended to the 200 mM potassium phosphate buffer (pH 7.0) with the same wet cell weight of 400 g l^{-1}, respectively.
[b]P, Protein concentration; [c]A, α-galactosidase activity; [d]I, invertase activity.

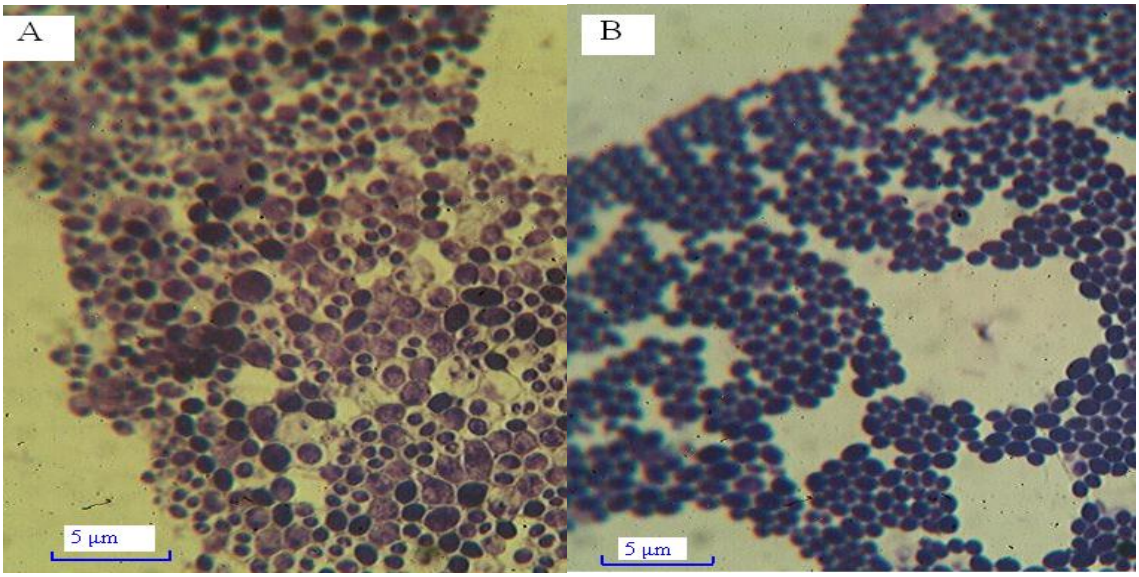

Figure 5. Microscopy of *S. cerevisiae* Z-06 cells. (A) Cells from the control. (B) Cells from the mixed culture.

of that in the control. However, compared to biomass of *S. cerevisiae* Z-06 obtained in the control, near 70% of which obtained in the mixed culture. From these results, it can be concluded that the *S. cerevisiae* was more favorable to compete for survival than that of the *A. oryzae* under stress condition.

In order to further determine the effect of mixed culture on the enzymes and protein synthesis of the *S. cerevisiae* Z-06, the yeast cells was disrupted and analyzed partially. As shown in Table 1, the protein content of the *S. cerevisiae* cells in the mixed culture was near 1.3-fold higher than that of the control. Nevertheless, the difference of the protein contents and the enzyme activities of the cell extract between the control and the mixed culture were insignificant. Therefore, in the mixed culture, the protein content of the yeast cell wall was enhanced, which resulting in significantly differences of the protein content and enzyme activities of the cell debris between the control and the mixed culture. These results also indicated that most of α-galactosidase and invertase production by *S. cerevisiae* Z-06 in the mixed culture were neither *in vitro* nor *in vivo*; adversely they were integral proteins which were not easily extracted from the membranes of the yeast cells (Prescott et al., 1999).

Effect of mixed culture on morphological and physiological characteristics of *S. cerevisiae* Z-06

To investigate the effect of mixed culture on the growth performance of *S. cerevisiae* Z-06, the yeast cells was dyed by crystal violet and examined using biological microscope. Interestingly, the observation revealed that the yeast cells from the mixed culture were stained more strongly than that of the cells from the control, and similar phenomena were found when the cells were dyed using safranin and other dyes (Figure 5). Further observation found that the yeast cells from the mixed culture were round

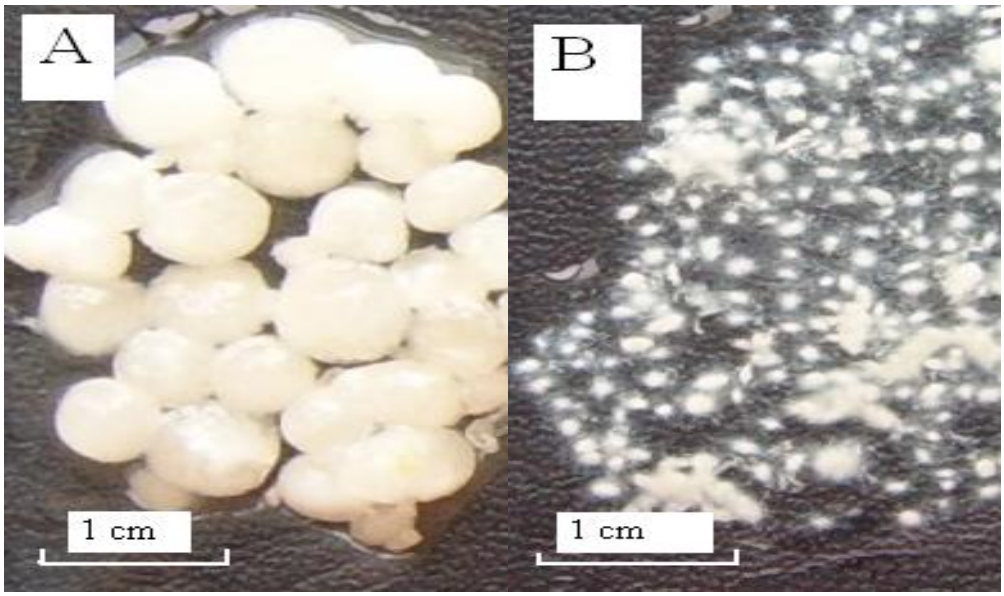

Figure 6. Mycelia pellets of *A. oryzae* L-09 from the control (A), and that from the mixed culture (B).

with an average width of 1.0 μm; meanwhile, the cells from the control were oval with an average width of 1.3 μm. Nevertheless, the average width of mycelia pellets from the control was 0.5 cm, which was 5-fold higher than that of the mycelia pellets from the mixed culture (0.1 cm) (Figure 6). This suggests that in the poor-nutrient, to compete for the survival, the cell wall of the yeast in the mixed culture was improved, which was more favorable for uptake *in vitro* substrate than that of the *A. oryzae* cell.

Ethanol production from soybean sirup in the simultaneous saccharification and fermentation process

To convert soybean syrup to ethanol economically, optimum fermentation conditions were used to maximize the ethanol productivity. The liquefaction of was completed in 24 h, however, the residual glucose concentration was constantly very low through the fermentation process. This suggested that the saccharification speed of the starch just be equal to the consumption speed of the *S. cerevisiae*, which weakened the sugar repression significantly. Meanwhile, as the metabolism of the *A. oryzae* was restrained in this anaerobic procedure, the carbon source was utilized solely by the yeast for ethanol production; then high ethanol concentration of 15.2% (V/V) was obtained in 60 h, and the conversion efficiency of starch to ethanol was 96.3% of the theoretical ethanol yield.

DISCUSSION

Previous studies have shown that cultivated in stress condition resulting in a change in the component of the fungal plasma membranes (Maldonado and Strasser de Saad, 1998) and also the osmotic stress suppresses cell wall stiffening and change the component in wheat coleoptiles (Wakabayashi et al., 1997). This indicates that in the stress condition, the structure, component, and then function of the cell wall was significantly improved, which was critical for the surviving of the strain (Malgorzata et al., 2006). This phenomena was also been found in our studies, where the protein concentration of the cells from the mixed culture was enhanced near 1.30-fold greater than the values of the cells obtained from the control. Considering the differences between the protein concentrations of the yeast cell extract from the mixed and the control culture were inconspicuous, the *in vivo* carbon and energy flux was redistributed to synthesize more protein in the cell wall which improved the potential of the cell wall dramatically.

It was previously found that α-galactosidase and invertase were inducible enzymes, and the synthesis of them was repressed by the monosaccharides. Therefore, to enhance the enzymes production, the concentration of the inducers should be increased, meanwhile, decreased the concentration of the corepressors (Prescott et al., 1999). In the mixed culture of *S. cerevisiae* Z-06 and *A. oryzae* L-09, the monosaccharides were utilized competitively, whose concentration fallen to 1% at 24 h of the culture, nevertheless, the concentration of the monosaccharides in the control was over 1% at 48 h of the culture. Further experiments showed that the adsorp-tion potential of the yeast cell wall was detectable improved in the mixed culture, which can further enforce the molecules of the inducer attaching the yeast cell membrane; therefore, the synthesis of the both enzymes was promoted significantly (Prescott et al., 1999; Turner et al., 2002). However, this phenomenon was not found in the

strain of *A. oryzae*, which may due to the different intrinsic biochemical, structural and genetic properties between the fungal mycelium and the yeast cell (Małgorzata et al., 2006). Our observation is also consistent with a previous study where the binding, as well as accumulating capacities for the metal of the yeast cell wall were improved under stress condition (Blackwell et al., 1995).

In order to enhance the α-galactosidase and invertase production of *S. cerevisiae* Z-06 in the submerged culture system, this strain was co-cultivated with *A. oryzae* L-09 at the beginning of the culture. As a result, α-galactosidase and invertase activities were enhanced over 9 and 15-fold greater than the values obtained in the cultures of single strain, respectively. Further studies found the mixed culture system influenced the component of the yeast cells wall, and the morphological as well as the physiological characteristics of the both strains significantly. By enhancement of the both enzymes production in the submerged culture using the mixed culture system, our investigation provides a novel way to regulate the cells growth and enzymes yields in the submerged culture system, which can be applied in the other microbial gene engineering strains for increasing inducible enzymes synthesis.

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