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Formation and Regeneration of protoplasts by Novozym 234 from *Kluyveromyces fragilis* N100 and *Candida pseudotropicalis* CBS607

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Novozym 234에 의한 *Kluyveromyces fragilis* N100과 *Candida pseudotropicalis* CBS607의 원형질체 형성과 재생

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ABSTRACT

Formation and regeneration of protoplasts by Novozym 234 from *Kluyveromyces fragilis* N100 and *Candida pseudotropicalis* CBS607 were studied. This enzyme was more effective on cells grown at exponential phase than those at stationary one to convert intact cells into protoplasts. As osmotic stabilizer, ammonium sulphate was suitable for not only protoplast formation but also regeneration in *K. fragilis* as well as in *C. pseudotropicalis*. Optimal enzyme concentraion was 3mg per ml in *K. fragilis* and 1~3mg per ml in *C. pseudotropicalis*, respectively. After the exposure of *K. fragilis* cells to 3mg per ml of enzyme for 3hr at 30°C, approximately 95% of protoplast formation of all observed cells was obtained, while about 100% from *C. pseudotropicalis* under the same condition was produced. The regeneration frequency of protoplasts by this enzyme was much lower than that by snail enzyme(Glusulase) although Novozym 234 converted cells from above two species into protoplasts free of cell debris effectively, compared with Glusulase. Novozym 234 appears to be suitable for subcellular fractionation to obtain nuclei or other organelles rather than protoplast regeneration.

INTRODUCTION

The formation and regeneration of protoplasts by Novozym 234 from several yeasts has been reported(Stephen and Nasim, 1981; Dickinson and Isenberg, 1982). This enzyme gave a high frequency formation of protoplasts in the following species of yeasts: *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactic*, *Trichosporon pullulans* and *Schwaniomyces alluvius*. The results so far reported showed that the production of protoplasts by this enzyme was comparable to that obtained using snail enzyme (Glusulase). The frequency of regeneration, however, varied with the species used and ranged between 10 and 18%. As compared with the results obtained using Glusulase(Svoboda, 1966; Nečas *et al.*, 1969; Sarachek *et al.*, 1981; Pesti *et al.*, 1982), this frequency value was much lower.

The formation and regeneration of protoplasts from *K. fragilis* and *C. pseudotropicalis* by Novozym 234 has not yet been undertaken. These two species have been recently investigated for commercial production of β-D-galactosidase since they have an ability of producing greater amount of the enzyme than other microorganisms(Aunthrup, 1977; Pedrique and Castillo, 1982). *C. pseudotropicalis* may be regarded as the imperfect form of *K. fragilis*(Lodder, 1970) and two species show a similar DNA base composition

(Stenderup and Bak, 1968; Bicknell and Douglas, 1970). The relationship between above two species could be a high probability of maintaining stability of fusion hybrid between them, and resultant construction of polyploid strains may be suitable for use of higher production of β-D-galactosidase on industry.

In the present study, first of all, the formation and regeneration of protoplasts from *K. fragilis* and *C. pseudotropicalis* by Novozym 234 was undertaken for the possibility of an increase in β-D-galactosidase through protoplast fusion between *C. pseudotropicalis* strains and between *K. fragilis* and *C. pseudotropicalis.*

MATERIALS AND METHODS

Media and enzymes used

All medium constituents were obtained from Difco Laboratories Detroit Michigan, U.S.A.. Novozym 234 (Novo industrias Novoalle. DK-2880 Bagsvoerd, Denmark) was generously gifted from Novo industrias and Glusulase (Sulfatase/β-glucuronidase) was obtained from Sigma Chemical Company. The yeast strains used here were *Kluyveromyces fragilis* N100 and *Candida pseudotropicalis* CBS607.

Formation of protoplasts

The culture medium used here was YEPD which was consisted of 3% (w/v) glucose, 0.5% (w/v) peptone and 0.5% (w/v) yeast extract. This medium was used throughout all experiments unless otherwise stated. To obtain exponential cells, two species were grown in 50ml of YEPD at 30°C for 8 to 16 hr on reciprocal shaker (120 strokes per min). Cells were harvested by centrifugation at 1000×g for 3min, then cell suspension was diluted to $2\sim4\times10^7$ per ml of distilled water. The method for the formation of protoplasts was used here with minor modification of method outlined by Kuo and Lampen (1971). Prior to the treatment with Novozym 234, the cells were washed once with distilled water, twice with 5mM $MgSO_4$ and 5mM citrate-phosphate buffer (pH6.0) containing 0.5M $(NH_4)_2SO_4$, 0.75M NaCl, 0.6M sorbitol and 0.6M KCl respectively as osmotic stabilizer. To 5ml of above cell suspension, the following suspensions were added: 0.05M tris-HCl (pH 6.0) 1ml, 1.2M KCl containing 0.02M $MgSO_4$ 6ml, 1M 2-mercaptoethanol 0.2ml and 0.01M citrate-phosphate buffer (pH5.85) containg 0.6M KCl 1ml. After the reaction mixture was incubated at 30°C for 30 min on reciprocal shaker (120 strokees per min), five different concentrations of Novozym 234 (i.e., 1, 2, 3, 4.5, 6mg per ml) were added and reacted at 30°C during the period from zero time to 3 hr. The number of protoplasts formed was counted under phase contrast microscope (Nikon Multipurpose microscope, Nippon Kogaku K.K., Japan), using haemacytometer.

Regeneration of protoplasts

The method of Fukui *et al.* (1969) was modified for the protoplast regeneration. Protoplasts were suspended at $1.5\sim2\times10^4$ per ml in 5mM citrate-phosphate buffer (pH6.0) containing 0.75 M $(NH_4)_2SO_4$ as osmotic stabilizer and 20mM 2-(N-morpholino) ethane sulfonic acid. 0.1ml of this suspension was put on the solid plates containing 5mM citrate-phosphate buffer (pH6.0), 2% agar and 0.5M $(NH_4)_2SO_4$, and was smeared with bent glass rod. Sabouraud agar with 0.5% yeast extract, 0.5M $(NH_4)_2SO_4$ and vitamin complex (Wickerham, 1951) was overlayed on the smeared plates and then embedded protoplasts were incubated at 30°C and/or 37°C for 48~96 hr until colonies developed.

RESULTS AND DISCUSSION

Effect of Novozym 234 on the cell lysis,

When *Kluyveromyces fragilis* cells were digested in protoplasting solution with 3mg Novozym 234 per ml, the percentage of cell lysis

of total cell number was at the lowest value of 8% during an early log phase(8hr), with a rapid increase in cells grown for 9~12hr(25~72%), and reached the maximum of 80% at 24 hr which became stationary phase. Similarly, *C. pseudotropicalis* exhibited 15% at an early log phase (10hr) and 80% at stationary phase (24hr), respectively (Fig. 1). These indicate that cell lysis occurs much more at stationary phase than at exponential phase. Similar

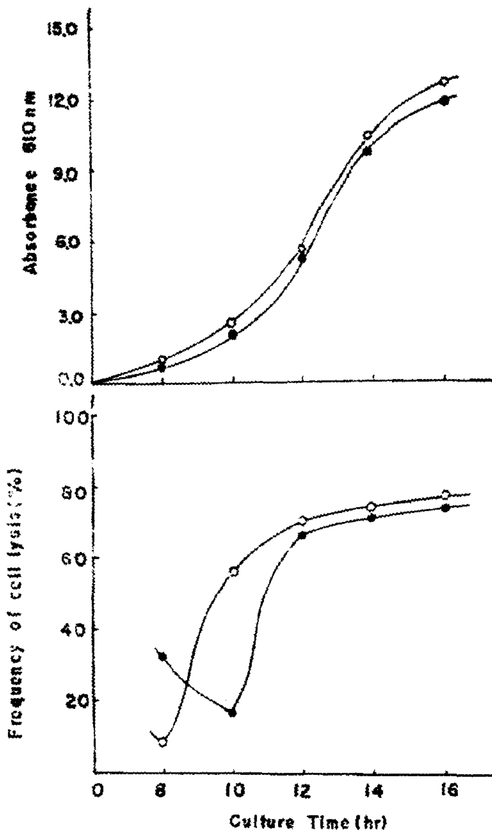

Fig. 1. The growth curve and effect of Novozym 234 on the cell lysis according to time dependent growth.

Yeast cells were removed at the indicated time of culture in YEPD for 8~16hr. The percentage lysis of cells was determined by counting the remaining protoplasts under phase contrast microscope (×500). The composition of reaction mixture and temperature were the same as described in text. Osmotic stabilizer was 0.5M $(NH_4)_2SO_4$. Enzyme concentration and exposure time were 3mg per ml and 3hr, respectively. Symbols: *K. fragilis*; ○—○, *C. pseudotropicalis*; ●—●

result was reported by Dickinson and Isenberg (1982), who found that approximately 50% of total cells in *Schizosaccharomyces pombe* was lysed at stationary phase, but cell lysis at log phase was negligible. However, the cells of *K. fragilis* and *C. pseudotropicalis* appear to be more susceptible to Novozym 234 than *Sch. pombe*, which may be caused by somewhat difference in the composition of cell wall between former two budding yeasts and latter fission one. Schwenke and Nagy(1978) showed that, unlike the situation in the majority of other budding yeasts, *Sch. pombe* has a particular composition of cell wall which was resistant to Glusulase(Streiblova, 1968). Since the composition of Novozym 234 is different from that of Gluslase in that former enzyme has α-1, 3-glucan glucanohydrolase, neutral protease and a minor amount of cellulase(Budtz-Jörgensen and Kelstrup, 1977), it is possible that there may be different effect on the cell lysis.

Effect of osmotic stabilizers on the formation of protoplasts

As osmotic stabilizer for the protplast formation frnm *K. fragilis* and *C. pseudotropicalis*, $(NH_4)_2SO_4$ and NaCl were better to prevent osmotic lysis than sorbitol and KCl, and optimal concentration were 0.5M and 0.75M, respectively(Fig. 2). 0.6M sorbitol and 1.2M sorbitol have been used as osmotic stabilizer in *Sch. pombe* (Dickinson and Isenberg, 1982; Stephen and Nasim, 1981). Generally, sugars and sugar alcohols have proved more effective on yeasts when compared with inorganic salts(Peberdy, 1979). However, inorganic salts such as $(NH_4)_2$-SO_4 and NaCl are more effective than sugar alcohol(sorbitol) on *K. fragilis* and *C. pseudotropicalis*, confirmed by Rost and Venner(1975) that $(NH_4)_2SO_4$ was suitable for the protoplast formation from *K. fragilis* when treated by Glusulase.

Effect of enzyme concentration on the formation of protoplasts

Optimal concentration of Novozym 234 in *K*.

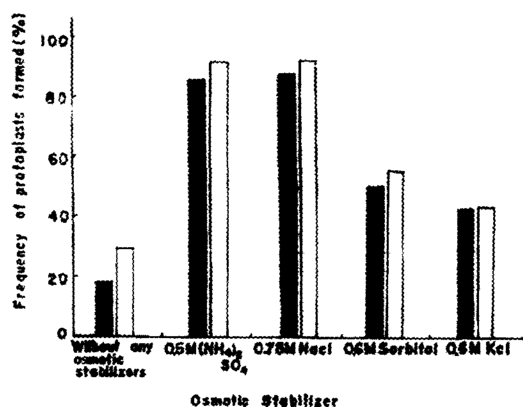

Fig. 2. The effect of osmotic stabilizers on the formation of protoplasts

K. fragilis and *C. pseudotropicalis* were grown in YEPD for 8 and 10hr, respectively. The pretreatment of cells with four different osmotic stabilizers was made to compare the degree of protoplast formation. The protoplasts formed of total cells were calculated. The other procedures except for osmotic stabilizers were the same as described in Fig. 1.

Symbols: *K. fragilis*; □, *C. pseudotropicalis*; ■

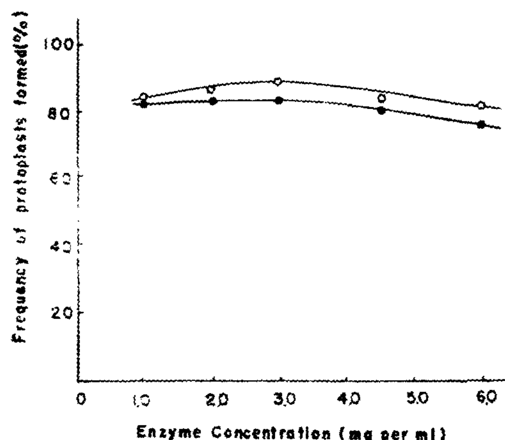

Fig. 3. The effect of enzyme concentration on the formation of protoplasts

The culture time of *K. fragilis* and *C. pseudotropicalis* was the same as described in Fig. 2. The cells removed were exposed with five different concentration of enzyme. The other procedures except for enzyme concentration were the same as described in Fig. 1.

Symbols: *K. fragilis*; ○—○, *C. pseudopicallis*; ●—●

fragilis was 3mg per ml at which concentration incubation for 3 hr at 30°C yielded approximately 90% of protoplast formation of total cells. This frequency gradually decreased at lower or higher concentration than 3 mg per ml (Fig. 3). However, *C. pseudotropicalis* gave the protoplast formation between 83 and 85% with similar rate at 1~3 mg per ml while showing a small decrease in frequency of protoplast formation at higher concentration than 3 mg per ml (Fig. 3). Although enzyme concentration may vary with exposure time, culture age, yeast species and osmotic stabilizer used, the present result indicates that the enzyme concentration of 3mg per ml was appropriate to obtain the protoplasts from *K. fragilis* and *C. pseudotropicalis* at the above reaction condition.

Effect of enzyme exposure time on the formation of protoplasts

When 3mg Novozym 234 per ml was exposed to exponentially grown cells of *K. fragilis* at 30 min of interval, protoplast formation was rapidly induced at 30 min, which became about

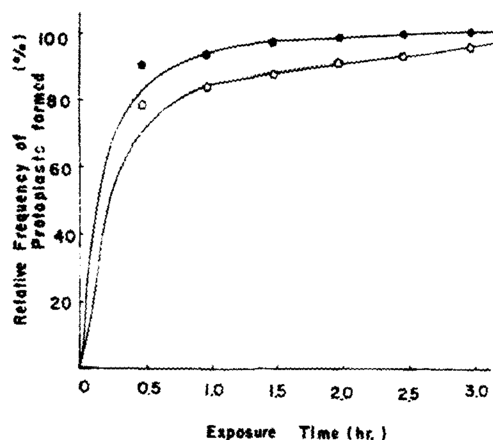

Fig. 4. The effect of enzyme exposure time on the formation of protoplasts

The culture time of *K. fragilis* and *C. pseudotropicalis* was the same as described in Fig. 2. Yeast cells were removed at 30 min of interval from reaction mixture. The relative percentage of protoplasts formed was measured from the observed cells on the stage of microscope. The other procedures except for exposure time were the same as described in Fig. 1.

Symbols: *K. fragilis*; ○—○, *C. pseudotropicalis*; ●—●

80% of the observed cells (Fig. 5a), and thereafter slowly increased, reaching maximum yield of 95% at 3 hr (Fig. 5b), which showed hyperbolic curve (Fig. 4). On the other hand, *C. pseudotropicalis* exhibited 92% at 30 min, showing a more rapid formation of protoplasts than *K. fragilis*, and reached about 100% after 3hr (Fig. 5c,d). Similar result was reported by Stephen and Nasim (1981), who showed that the frepuency of protoplast formation ranged between 80 and 90% when five species including *Sch. pombe* were treated with 2mg per ml for 30min. Dickinson and Isenberg (1982) reported that the conversion of all observed cells to protoplasts had been made after 3.5 hr using 3mg per ml, which was nearly identical with the result obtained here except for 0.5hr longer exposure.

The comparison of Novozym 234 with Glusulase on the formation and regeneration of protoplasts

When Novozym 234 and Glusulase were compared on the formation of protoplasts from two species, the cells treated with Novozym 234 were more lysed than those with Glusulase (Table 1). Howover, not only the percentage formation of protoplasts of all observed cells were greater in Novozym 234, but also protoplasts obtained were more free of cell debris than in Glusulase (Fig. 5 e,f). As shown in five different yeasts (Stephen and Nasim, 1981) and *Sch. pombe* (Dickinson and Isenberg, 1982), Novoxym 234 may be used to obtain protoplasts, nuclei and other organelles for the study of subcellular structure of *K. fragilis* and *C. pseudotropicalis*.

To regenerate the protoplast wall, protoplasts produced by Novozym 234 treatment after stabilization of cells with 0.5M $(NH_4)_2SO_4$ were resuspended in higher osmolarity (0.75M $(NH_4)_2SO_4$) to give a greater protection of protoplasts with no leakage of intracellular material, then were transferred on solid plate (regeneration medium) containing 0.5M $(NH_4)_2SO_4$. It is noteworthy that the maintenance of lower osmolarity in regeneration medium than in resuspending solution of protoplasts was more effective. This was confirmed by Kuo and Lampen (1971) who found that the synthetic activity of protoplasts in *Saccharomyces* 1016 was greatly enhanced in lower osmolarity. It is notable that NaCl exhibited the same effect on the protoplast formation as $(NH_4)_2SO_4$ but deleterious result on the regeneration (Table 2).

The regeneration frequency of protoplasts formed by Novozym 234 was much lower when compared with that by Glusulase. The frequency was comparable to the result (10~18%) obtai-

Table 1. The comparison of Novozym 234[a] with Glusulase[b] on the formation of protoplasts

Yeast species	Frequency of cell lysis (%)		Relative frequency of protoplast formed (%)	
	Novozym	Glusulase	Novozym	Glusulase
K. fragilis (N 100)	8~10	3~ 5	93~ 96	90~92
C. pseudotropicalis (CBS 607)	10~15	5~10	98~100	90~93

a. The concentration of Novozym 234 was 3mg per ml.
b. The concentration of Glusulase was the same method as described in Kuo and Lampen (1971).
The other procedures except for 3h reaction were the same as described in Fig. 4.

Table 2. The comparison of regeneration of protoplasts formed by Novozym 234 with those by Glusulase

Yeast species	Regeneration frequency of protoplasts (%)	
	Novozym 234	Glusulase
K. fragilis N 100	10~15[a]	70~75[a]
	(2~5)[b]	(15~20)[b]
C. pseudotropicalis	14~20[a]	75~90[a]
CBS 607	(3~7)[b]	(20~35)[b]

The procedures were described in the text.
a : Regeneration medium containing 0.5M $(NH_4)_2SO_4$
b : Regeneration medium containing 0.5M NaCl

ned by using Novozym 234 (Stephen and Nasim, 1981). It should be, however, noted that Novozym 234 was not effective in the regeneration of protoplasts from *K. fragilis* and *C. pseudotropicalis* because of the great difference between the result presented here by Novozym 234 (Table 2) and those obtained by many workers, who showed that the percentage of regeneration of protoplasts by Glusulase ranged between 20 and 70% in *Candida albicans* (Saracheck et al., 1981; Pesti and Ferenczy, 1982), and gave 50~70% in *Saccharomyces cerevisiae* (Svoboda, 1966) and 90% in *Sch. pombe* (Nečas *et al.*, 1968), respectively.

摘 要

Novozym 234에 의한 *K. fragilis*와 *C. pseudotropicalis*의 원형질체 형성과 재생에 관한 연구를 하였다. intact cell을 원형질체로 전환시키는데 정지기의 세포보다 대수기에 있는 세포가 효과적이었으며 osmotic stabilizer로는 $(NH_4)_2SO_4$가 두 종 똑같이 원형전체 형성이나 재생에 있어서 적합했고 최적 효소농도는 *K. fragilis*에서는 3mg/ml, *C. pseudotropicalis*에서는 1~3mg/ml이었다. 반응 후 관찰된 세포 중의 원형질체 형성 수율은 *K. fragilis*에서 약 95%, *C. pseudotropicalis*에서 약 100%였으나 원형질체의 재생은 Glusulase와 비교해 볼 때 매우 낮았다. 그러나 세포 이물질이 없는 원형질체를 얻는데는 Novozym 234가 적합한 것 같다.

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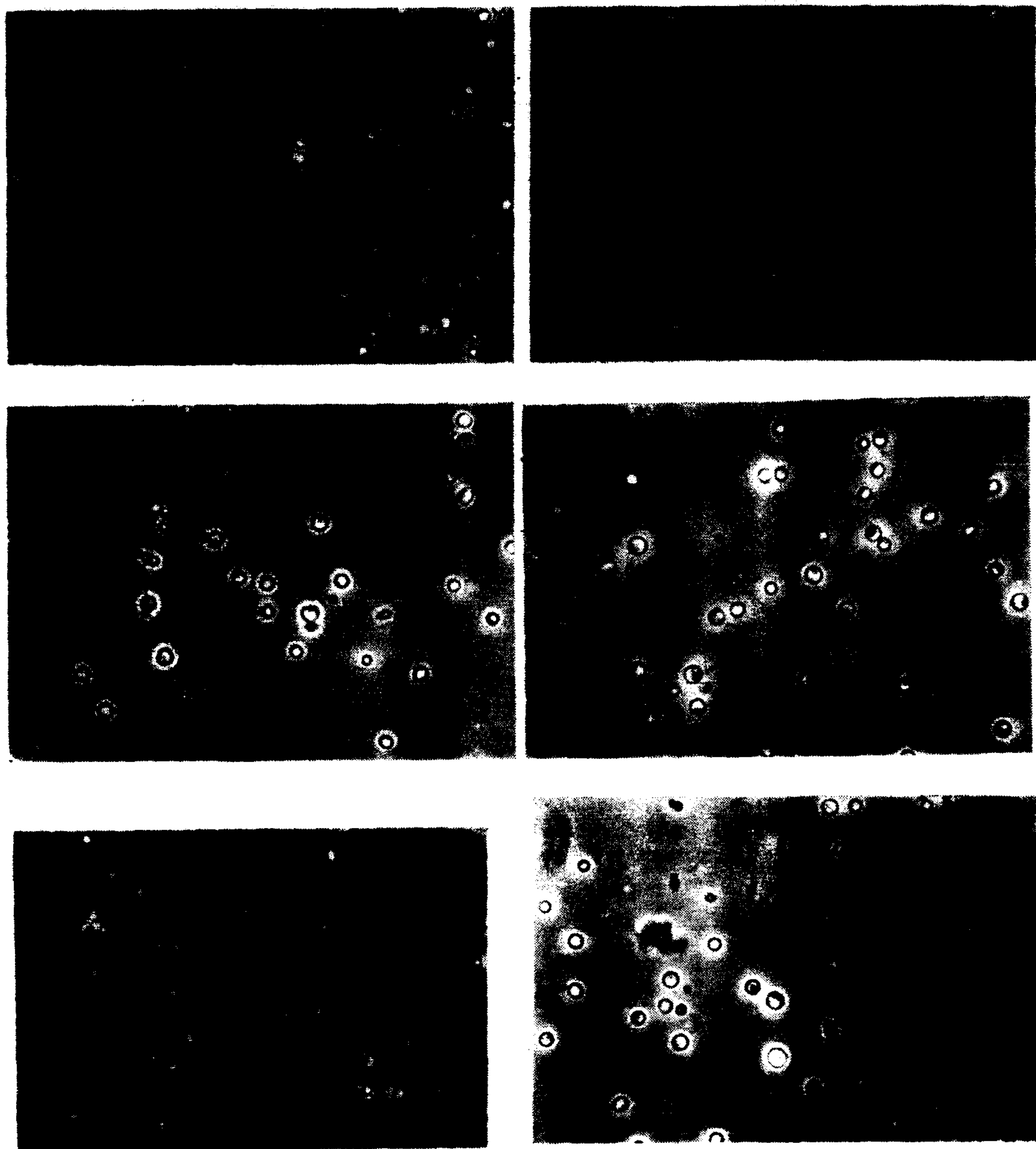

Fig. 5. Protoplast formation from *K. fragilis* (a, b, e) and *C. pseudotropicalis* (c, d, f). The cells were digested with 3mg per ml of Novozym 234 at 30°C for 30 min (a and c) and 3hr (b and d), and with Glusulase for 3hr (e and f). ×500.