Indian Journal of Biochemistry & Biophysics
Vol. 44, April 2007, pp. 76-81

# Purification and properties of α-galactosidase from white-rot fungus *Pleurotus florida*

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*Received 26 June 2006; revised 15 February 2007*

α-Galactosidase was strongly induced in the white-rot fungus *Pleurotus florida* by arabinose than its natural substrates and was purified to homogeneity by acetone precipitation, ultrafiltration and DEAE-Sepharose chromatography. The enzyme was a monomeric protein with a molecular mass of ≅ 99 kDa, as revealed by native-PAGE and SDS-PAGE. α-Galactosidase was optimally active at 55°C for the hydrolysis of *p*-nitrophenyl-α-galactopyranoside (PNPαG) and lost its 20% and 50% of original activity in 30 min at 60°C and 70°C, respectively. The *p*H optimum of the enzyme was between 4.6 and 5.0. It was stable in a wide *p*H range (*p*H 4.0 to 9.0) at 55°C for 2 h. The $Ag^{+}$ and $Hg^{2+}$ strongly inhibited the enzyme activity. Galactose, glucose, maltose and lactose also inhibited the enzyme activity, whereas N-bromosuccinimide treatment resulted in near total loss of acitivity. The $K_m$ and $V_{max}$ values of the enzyme for PNPαG were found to be 1.1 m*M*, and 77 μmol $min^{-1}$ $mg^{-1}$, respectively. α-Galactosidase immobilized in agar was more effective for the degradation of raffinose than in the sodium alginate. TLC results indicated its potential for the removal of raffinose and stachyose in soymilk.

**Keywords**: *Pleurotus florida*, α-Galactosidase, Purification, Kinetic studies, Soymilk, Immobilization, Agar

α-Galactosidase (α-D-galactoside galactohydrolase; EC 3.2.1.22) catalyzes the hydrolysis of α-1,6 glycosidic linkages of disaccharides (melibiose), oligosaccharides (raffinose) and polysaccharides (galactomannan)[1,2]. α-Galactosidase obtained from hyperthermophilic microorganisms has shown potential biotechnological applications in hydraulic fracturing of oil and gas wells[3]. Hyperthermostable and thermoactive hemicellulases, such as α-galactosidase and endo-β-1,4-mannanase are ideal candidates which can withstand the elevated temperature prevailing in the deeper reaches of the well-bore. They act on a viscous and water-soluble guar galactomannan which are flooded in the well-bore for stimulation[4]. Endo-β-1,4-mannanase act on interior mannan backbone while α-galactosidase liberate side-chain galactose. In the pulp and paper industry, endo-β-1,4-mannanase and α-galactosidase without cellulase are used to improve the bleaching of softwood pulp[5].

The mature seeds including those of soybean contain raffinose-family of oligosaccharides (RFO) as the storage carbohydrates (2-10%)[2,6,7]. Lactic acid bacteria (LAB) and fungi have successfully been used to remove the beany flavor and RFO in soymilk[8]. Invertase-free α-galactosidase has been found to be useful in beet sugar refining to increase the yield of sucrose[9]. α-Galactosidases have been reported from a variety of bacterial, yeast, fungal and plant sources[1,10-18]. To our knowledge, there are no reports on the purification, characterization and applications of α-galactosidase from the edible mushroom *Pleurotus* spp. In this communication, we report the purification and characterization of α-galactosidase from the white-rot fungus *Pleurotus florida.*

## Materials and Methods

### Source of enzyme

*Pleurotus florida* in the form of spawn culture was procured from the Department of Plant Pathology, Tamilnadu Agricultural University, Coimbatore, Tamilnadu, India. The purity of the fungus in spawn culture was checked and maintained on Czapek Dox agar slants. The spawn culture was dissolved in acetate buffer (0.1 *M*, *p*H 4.6) and was filtered through a Whatmann filter paper. The filtrate was centrifuged at 5000 × *g* and 10°C for 30 min. The supernatant served as source of enzyme and stored at 4°C.

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**Effect of sugars on enzyme induction**

Induction of α-galactosidase in *P. florida* was studied by using arabinose, xylose galactose, glucose, fructose, lactose, maltose, sucrose, xylan, guar gum, and starch as sole source of carbon in Czapek Dox broth. Batch submerged fermentation was carried out for 5 days on an orbital shaker at 120 rpm and 30°C and the mycelia were removed by filtration. The filtrate was checked for protein and α-galactosidase activity[18,19].

**Assay of α-galactosidase**

Assay of α-galactosidase was carried out by incubating 100 µl of an appropriately diluted enzyme, 800 µl of acetate buffer (0.1 *M*, *p*H 4.6) and 100 µl of 10 m*M* *p*-nitrophenyl-α-galactopyranoside PNPαG (Sigma) for 15 min at 55°C. The reaction was stopped by the addition of 3 ml of 0.2 *M* $Na_2CO_3$ and the absorbance of *p*-nitrophenol liberated was measured at 405 nm[18]. One unit of enzyme activity was defined as the amount that liberated one micromole of product per min under the assay conditions.

**Purification of enzyme**

The crude enzyme supernatant at 4°C was added to chilled acetone (at −20°C) in a ratio of 1:1 (%, v/v) with agitation and the mixture was placed aside without agitation for 12 h at 4°C. The precipitate obtained was dissolved in a small volume of acetate buffer (50 m*M*, *p*H 4.6) and dialyzed. The dialyzate was centrifuged at 10, 000 × *g* and 4°C for 30 min. The clear supernatant thus obtained containing α-galactosidase was subjected to ultrafiltration (PALL India Pvt. Ltd., 77200-52) using an Omega membrane of 50 kDa. The retentate and filtrate were collected separately and centrifuged at 4, 500 × *g* and 4°C. Retentate from ultrafiltration was loaded on to a column (2.5 × 12 cm) of DEAE-Sepharose, previously equilibrated with acetate buffer (25 m*M*, *p*H 4.6). The enzyme was eluted with a linear gradient of 0.5 *M* NaCl at a flow rate of 30 ml $h^{-1}$ and 3 ml fractions were collected. All the fractions were estimated for α-galactosidase activity and protein at 280 nm. The active fractions having high α-galactosidase activity were pooled and dialyzed and the dialyzate was used for further experiments.

**Molecular mass determination**

Native-PAGE and SDS-PAGE were carried out by the method as described by Laemmli[20] with 8% polyacrylamide slab gel and the protein bands were visualized by silver staining. Marker proteins (29-205 kDa, Genei, India) were run in a parallel lane. The molecular mass of α-galactosidase was estimated from its position relative to the standard proteins.

**Effect of *p*H and temperature**

The optimum *p*H for α-galactosidase activity was determined by varying the *p*H ranging from 3.6 to 9.0. The various buffers used to monitor the effect of *p*H on the enzyme activity were: 100 m*M* acetate buffer (*p*H 3.6 to 5.5), 100 m*M* sodium phosphate buffer (*p*H 6.0 to 7.0) and 100 m*M* Tris-HCl buffer (*p*H 7.5 to 9.0). The optimum temperature was determined at varying the temperatures from 20 to 70°C. The *p*H stability was determined by incubating the enzyme at *p*H 4.0, 5.0, 6.0, 6.5, 7.0, 8.0 and 9.0 for 2 h and the residual activity was determined at an interval of 15 min. For studying the time course of thermal inactivation, the enzyme in acetate buffer (50 m*M*, *p*H 4.6) was pre-incubated at temperatures 60°C and 70°C for 30 min and 1 h and the residual activity was determined at an interval of 15 min.

**Effect of metal ions, sugars, reagents and substrate concentration**

Enzyme assays were performed in the presence of various metal ions (final concentration, 10 m*M*) such as $K^+$, $Ca^{2+}$, $Hg^{2+}$ and $Fe^{2+}$ as chloride salts, $Ag^+$ as nitrate salt, $Zn^{2+}$, $Cu^{2+}$ and $Mg^{2+}$ as sulphate salts. Sugars such as xylose, glucose, galactose, fructose, maltose, lactose, melibiose and raffinose (final concentration 5 m*M*) and some reagents [sodium azide, EDTA, 1,10-phenanthroline, N-bromosuccinimide (NBS) and PMSF, final concentration, 5 m*M*] were incubated for 15 min at 37°C, prior to addition of substrate PNPαG. The relative activity of the enzyme was calculated by comparison of its activities in the presence and absence of the reagent. Michaelis-Menten constant ($K_m$) and the reaction rate ($V_{max}$) for synthetic substrate PNPαG was carried out in the range of 0.1 to 1 m*M*.

**Soymilk treatment**

Soymilk was prepared by the method of Mulimani and Ramalingam[18]. The 5 ml of soymilk was added to 1 ml of enzyme (6 units) and incubated at *p*H 5.5 and 55°C for 2 h. Every 30 min intervals, 1 ml of mixture was withdrawn, to which 0.2 ml of 0.2 *M* $ZnSO_4$ and 0.18 *M* $Ba(OH)_2$ were added to precipitate the proteins[21].

**Degradation of raffinose by immobilized α-galactosidase**

The 2 ml of enzyme solution (11 units $mg^{-1}$) was added with a sterile (10 ml) alginate (2.5% w/v) and then dropped into a sterile $CaCl_2$ solutions (0.1 $mol^{-1}$) to form beads. The average biocatalyst bead diameter was 0.3 cm. Similarly, 2 ml of the enzyme solution (11 units $mg^{-1}$) was added to 10 ml of molten agar (2.5%) at 45°C and the mixture was allowed to solidify at 37°C. Stainless mesh was used to make the agar cubes approximately 0.3 cm size. The beads were washed thoroughly with water and 25 ml of raffinose solution (1%, w/v) was added separately to each form of immobilized beads in a Erlenmeyer flasks (250 ml). The flasks were then placed on an orbital shaker at 37°C for 2 h and the contents were analyzed for reduction in the levels of raffinose[21].

## Results and Discussion

**Enzyme induction**

Table 1 shows the effect of sugars on induction of α-galactosidase from *P. florida*. Among the various sugars tested, arabinose had a pronounced effect, whereas xylose, glucose, lactose and maltose had only a marginal on the enzyme induction. α-Galactosidase induction by polysaccharides (guar gum and xylan) was higher than simple sugars. Galactose, lactose, melibiose and raffinose induced α-galactosidase secretion in fungus *Mortierella vinacea*[22,23]. These sugars, except lactose also induced α-galactosidase in fungus *Gibberella fujikuroi* and bacterium *Corynebacterium murisepticum*[18,24]. Melibiose, a natural inducer of α-galactosidase in microbial sources failed to induce α-galactosidase in filamentous fungi and yeast-like fungi[25]. Incorporation of guar gum as the carbon source strongly induced α-galactosidase in *Penicillium ochrochloron*[26].

**Purification of α-galactosidase**

The α-galactosidase from culture filtrate of *P. florida* was purified to homogeneity with a yield of 0.17%. The culture supernatant contained 1.13 U of the enzyme activity mg $protein^{-1}$. After ultrafiltration of the enzyme through the filter with 50 kDa exclusion size membrane, the enzyme activity was found only in the retentate and not in the filtrate, This suggesting that the molecular mass of the enzyme was higher than 50 kDa. The results in Table 2 showed that α-galactosidase was purified to 36-fold. After three purification steps, native-PAGE and SDS-PAGE of the final enzyme preparation showed a single band. The molecular mass of the purified enzyme as estimated by SDS-PAGE was found to be ≅ 99 kDa (Fig. 1).

α-Galactosidase from *Ganoderma lucidum* consisted of four subunits each with a molecular mass of 56 kDa, as determined by SDS-PAGE[13]. The molecular mass of recombinant α-galactosidase I and II from *M. vinacea* was found to be 58-67 kDa and 51-58 kDa[9], respectively, whereas the molecular mass of the α-galactosidase from the thermophilic fungus *Humicola* sp. and *Aspergillus oryzae* was reported to be 87.1 kDa[11] and 66 kDa[15], respectively.

Table 1—Effect of sugars and some polysaccharides on the induction of α-galactosidase in *Pleurotus florida*

| Sugar/polysaccharide (2%, w/v) | Enzyme activity (U) |
|---|---|
| Arabinose | 0.124 |
| Xylose | 0.015 |
| Galactose | 0.053 |
| Glucose | 0.021 |
| Fructose | 0.037 |
| Lactose | 0.013 |
| Maltose | 0.015 |
| Sucrose | 0.033 |
| Guar gum | 0.059 |
| Starch | 0.034 |
| Xylan | 0.084 |

Table 2—Purification of α-galactosidase from culture filtrate of *P. florida*

| Enzyme fraction | Volume (mL) | Protein conc. (mg) | Activity (Units) | Specific activity (U/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|---|
| Culture filtrate | 55 | 99 | 112.75 | 1.13 | 1 | 100 |
| Acetone precipitation | 20 | 12 | 23.80 | 1.93 | 1.75 | 7.67 |
| Ultrafiltration | 11 | 9 | 34.42 | 3.82 | 3.38 | 6.10 |
| DEAE-Sepharose chromatography | 5 | 0.05 | 2 | 41 | 36 | 0.17 |

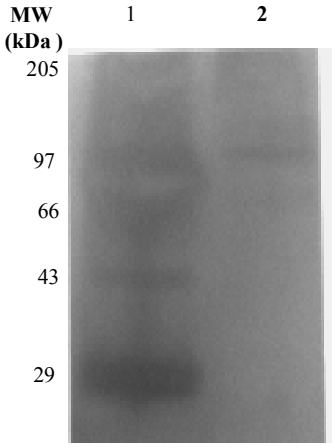


Fig. 1—Native-PAGE of purified enzyme from *P. florida* [Lane 1, molecular weight markers; and lane 2, purified sample]

**Effect of *p*H and temperature**

The enzyme activity at various *p*H and temperatures is shown in Fig. 2A and B respectively. The optimum activity was observed at *p*H 4.6, with PNPαG as the substrate. The enzyme was stable at *p*H 4.0-8.0 for 2 h and retained all its activity at *p*H 9.0 for 2 h, but lost its 40% activity in 3 h. α-Galactosidase from *Humicola* sp. showed its optimal activity at *p*H 5.0 and was stable in the *p*H range 4.5-6.5[11] whereas from *G. lucidum* had an optimum activity at *p*H 6.0[13]. Most fungal α-galactosidases exhibited their optimum activity between 4.5 and 5.5[27, 28].

α-Galactosidase from *P. florida* had an optimum temperature at 55°C (Fig. 2B). The enzyme activity increased with temperature up to 55°C and then decreased suddenly. The enzyme was stable up to 1 h at 55°C, but lost its 20% and 50% of its original activity in 30 min at 60°C and 70°C, respectively. The thermostability of α-galactosidase from *P. florida* was in agreement with that reported from fungal sources[11,13,15]. This property has an added advantage in food processing industries to minimize the contamination caused by mesophilic micro-organisms.

**Effect of metal ions, sugars and other chemicals**

The $K^+$, $Fe^{2+}$ and $Mg^{2+}$ had no effect on the α-galactosidase activity (data not shown), whereas $Fe^{2+}$ and $Mg^{2+}$ slightly enhanced the enzyme activity. However, the enzyme activity was inhibited to 85% and 95% up on addition of $Hg^{2+}$ and $Ag^+$, respectively, while, $Zn^{2+}$and $Ca^{2+}$ reduced the enzyme activity only slightly (less than 10% of control). $Ag^+$ and $Hg^{2+}$ completely inhibited α-galactosidase from

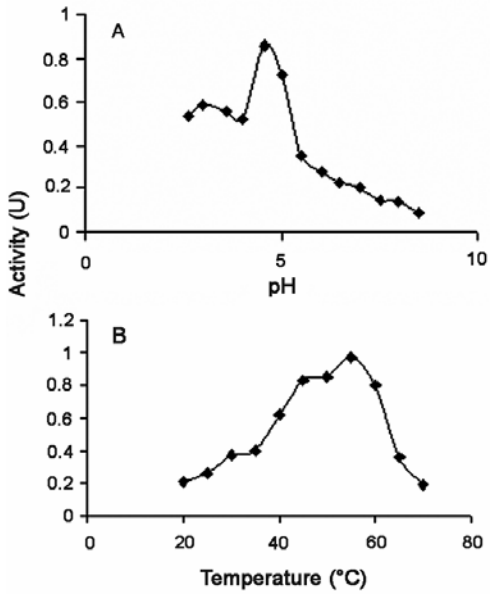


Fig. 2—Effect of *p*H (A) and temperature (B) on the activity of α-galactosidase from *P. florida*

alkalophilic strains of *Micrococcus* sp. and *Bacillus* sp[29]. These ions also inhibited α-galactosidases from plant (*Cucurbita pepo*) and fungal sources (*Torulaspora delbrueckii* IFO and *G. lucidum)*[13,30,31].

Among the sugars tested, galactose and maltose at 5 m*M* level showed more than 70% inhibition on α-galactosidase acitvity from *P. florida*. The increasing concentrations of galactose at 10, 25 and 50 m*M* levels showed an increase in the percent inhibition of enzyme activity (data not shown). Glucose, xylose, lactose, melibiose and raffinose inhibited the enzyme activity to varying extent (data not shown). Fructose had no inhibitory effect on the activity of α-galactosidase. Most of the α-galactosidases from fungal sources were not inhibited by glucose[14,15], except that from fungus *Humicola* sp. which was inhibited by D-glucose as well as by D-galactose and D-mannose[11]. Galactose also strongly inhibited α-galactosidases from fungal sources, such as *G. lucidum*, *Pycnoporus cinnabarinus* and *A. oryzae*[13-15], but did not inhibit α-galactosidase from alkalophilic bacteria, such as *Micrococcus* sp. and *Bacillus* sp. However, glucose and sucrose inhibited their activity[29].

Addition of NBS strongly inhibited the enzyme activity, indicating the presence of tryptophan at or near active site of the enzyme[15]. NBS inhibited a variety of α-galactosidases from fungal sources including *Humicola* sp[11,15]. The reagents such as EDTA and 1, 10-phenanthroline did not inhibit the

enzyme activity, suggesting that the metal ion was not required for the enzyme activity. α-Galactosidases from *Lactobacillus fermentum* and *G. lucidum* were not inhibited by EDTA[13,32].

**Kinetic parameters**

The $K_m$ and $V_{max}$ values of the enzyme for PNPαG at *p*H 4.6 and 55°C were determined as 1.11 mM and 77 μmol $min^{-1}$ $mg^{-1}$. The $K_m$ value of the α-galactosidase from *P. florida* was in good agreement with other fungal sources, such as *A. flavipes* (1.89 mM)[33], *Torulaspora delbrueckii* (2.8 mM)[31], bacterial source *Saccharopolyspora erythraea* (0.65 mM)[34] and recombinant α-galactosidase from *Thermus brockianus* ITI360 (2.47 mM)[35]. The $K_m$ value of the enzyme from *C. murisepticum* for PNPαG at 37°C was 0.17 mM[24].

**Degradation of raffinose by immobilized α-galactosidase**

The α-galactosidase immobilized in calcium alginate (2.5%) and agar beads retained 71% and 85% activity, respectively. Results indicated that the enzyme immobilized in agar was more effective than alginate for degradation of raffinose, possibly due to the more enzyme load in the agar beads. α-Galactosidase in mycelial suspension and pellet forms of *M. vinacea* were employed to decompose raffinose in beet molasses[22,23]. Immobilized α-galactosidase from *A. oryzae* on eupergit-C exhibited higher activity and thermal stability as compared to soluble enzyme[36].

**Soymilk treatment**

Thin-layer chromatography (TLC) analysis of enzyme-treated soymilk showed that α-galactosidase completely hydrolyzed raffinose and stachyose in soymilk in 2 h (data not shown). α-Galactosidase from *Humicola* sp. was effective at *p*H 6.0 and 60°C for the hydrolysis of raffinose and stachyose of soymilk[37]. α-Galactosidase from *L. fermentum* completely degraded raffinose and stachyose in commercial soymilk at *p*H 5.2 or 6.5 at 37 in 7 h[38]. α-Galactosidase from germinating guar and *G. fujikuroi* had been successfully used to degrade raffinose and stachyose in soymilk[6,18]. Disrupted and undisrupted mycelia and immobilized α-galactosidase from *M. vinacea* were used to hydrolyze raffinose and stachyose of soymilk[39].

## Conclusion

*P. florida* has been found to be a rich source of α-galactosidase, which can be purified by simple purification steps. The enzyme may find application in food processing industries for the degradation of raffinose-family of oligosaccharides. Cloning of α-galactosidase gene from *P. florida* in a suitable vector, followed by its expression in an appropriate host would go a long way to meet the industrial requirement of this enzyme.

## References


1 Dey P M & Pridham J B (1972) in *Advances in Enzymology* (Meister A, ed), pp. 91-30, Academic Press Inc., New York
2 Peterbauer A & Richter A (2001) *Seed Sci Res* 11, 185-197
3 McCutchen C M, Duffaud G D, Leduc P, Petersen A R H, Tayal A, Khan S A & Kelly R (1996) *Biotechnol Bioeng* 52, 332-339
4 Clarke J H, Davidson K, Rixon J E, Halstead, Fransen M P, Gillest H J & Hazlewood G P (2000*) Appl Microbiol Biotechnol* 53, 661-667
5 Zeilinger S, Kristufek D, Arisan-Atac I, Hodits R & Kubicek C P (1993) *Appl Environ Microbiol* 59, 1347-1353
6 Shivanna B D, Ramakrishna M & Ramadoss C B (1989) *Process Biochem* 24, 197
7 Cristafaro E, Mottu F & Wuhrmann J J (1974) in *Sugars in Nutrition* (Sipple H L & McNutt KW, eds), pp. 313-316, Academic Press, New York
8 Mital B K & Steinkraus K H (1979) *J Food Protect* 42, 895-899
9 Shibuya H, Kobayashi H, Satiot, Kim W S, Yoshida S, Kaneko S, Kasamo K & Kusakabe I (1997) *Biosci Biotechnol Biochem* 61, 592- 598
10 Post D A & Luebke V E (2005) *Appl Microbiol Biotechnol* 67, 91-96
11 Kotwal S M, Khire J M & Khan M I (1999*) J Biochem Mol Biol Biophys* 3, 9-17
12 Gaudreault P E & Webb J A (1983) *Plant Physiol* 71, 662-668
13 Sripaun T, Aoki K, Yamamoto K, Tongkao D, & Kumagai H (2003) *Biosci Biotechnol Biochem* 67, 1485-1491
14 Ohtakara A, Mitsutomi M & uchida Y (1984) *J Biol Chem* 48, 319-1327
15 Ramalingam & Mulimani (2004) *J Microbiol Biotechnol*, 14, 863-867
16 Kestwal R M & Bhide S V (2005) *Indian J Biochem Biophys* 42, 156-160
17 Anisha G S & Prema P (2006) *Indian J Biotechnol* 5, 373-379
18 Mulimani V H & Ramalingam (1995) *Biochem Mol Biol Int* 36, 897-905
19 Lowry O H, Rosebrough N J, Farr A L & Randall R L (1951) *J Biol Chem* 193, 265-275
20 Laemmli U K (1970) *Nature* 227, 680-685
21 Tanaka M, Thananunkul T C, Lee T C & Chichester C O (1975) *J Food Sci* 40, 1087-1088
22 Suzuki H, Ozawa Y, Oota H & Yoshida H (1969) *Agric Biol Chem* 33, 501-513
23 Kobayashi H & Suzuki H (1972) *J Ferment Technol* 50, 625-632
24 Nadkarni, M A, Nair C K K, Pandey V N & Pradhan D S (1992) *J Gen Appl Microbiol* 38, 23-34

25 McKay A M (1991) *J Food Sci* 56, 1749-1750
26 Dey P M, Patel S & Brownleader M D (1993) *Biotechnol Appl Biochem* 17, 361-371
27 Ademark, P, Larsson F, Tjerneld F & Stalbrand H (2001) *Enzyme Microbiol Technol* 29, 441-448
28 Rios S, Pedregosa M, Monistrol I F & Laborda (1993) *FEMS Microbiol Lett* 112, 35-42
29 Akiba T & Horikoshi K (1976) *Agric Biol Chem* 40, 1851-1855
30 Gaudreault P R & Webb J A (1983) *Plant Physiol* 71, 662-668
31 Oda Y & Tonomura K (1996) *J Appl Bacteriol* 80, 203-208
32 Garro M S, de Valdez G F, Oliver G & de Giori G S (1996) *J Biotechnol* 45, 103-105
33 Ozsoy N & Berkkan (2003) *Cell Biochem Funct* 21, 387-389
34 Post D A & Luebeke V E (2005) *Appl Microbiol Biotechnol* 67, 91-96
35 Fridjonsson O, Watzlawick H, Gehweiler A, Rohrhirsch T & Mattes R (1999) *Appl Environ Microbio*l 65, 3955-3963
36 Hernaiz M J & Crout D H G (2000) *Enyme Microbiol Technol* 27, 26-32
37 Kotwal S M, Gote M M, Sainkar S R, Khan M I & Khire J M (1998*) Process Biochem* 33, 337-343
38 Garro M S, de Giori G S, de Valdez G F & Oliver G (1993) *J Appl Bacteriol* 75, 485-488
39 Thananunkul D, Tanaka M, Chichester C O & Lee T C (1976) *J Food Sci* 41, 173-175