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A New Lipase Isolated from Oleaginous Seeds from *Pachira aquatica* (Bombacaceae)

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Abstract A new lipase from seeds of *Pachira aquatica* was purified to homogeneity by SDS-PAGE obtaining an enzyme with a molecular weight of approximately 55 kDa. The purified lipase exhibited maximum activity at 40°C and pH 8.0, for an incubation time of 90 min. Concerning temperature stability, at the range from 4 to 50°C, it retained approximately 47% of its original activity for 3 h. The enzyme activity increased in the presence of Ca^{++} and Mg^{++}, but was inhibited by Hg^{++}, Mn^{++}, Zn^{++}, Al^{+++} and various oxidizing and reducing agents. The lipase was highly stable in the presence of organic solvents, and its activity was stimulated by methanol. The values of K_m and V_{max} were 1.65 mM and 37.3 μmol mL^{-1} min^{-1}, respectively, using *p*-nitrophenylacetate as substrate. The enzyme showed preference for esters of long-chain fatty acids, but demonstrated significant activity against a wide range of substrates.

Keywords Enzyme · Lipase · Seeds · *Pachira aquatica* · Hydrolysis

Introduction

Lipases (E.C.3.1.1.3) are enzymes that are able to catalyze fat hydrolysis at an oil–water interface. This reaction is reversible, and the enzyme also catalyzes the synthesis of esters and transesterification in microaqueous conditions. Lipases are common among living beings [1].

Lipases that are present in oilseeds help to hydrolyze the ester bonds of storage triacylglycerols, and it has been reported that with a few exceptions, lipase activity is absent in ungerminated (or dormant) seeds and increases rapidly in post-germination [2]. In those cases where the enzyme is active in the dormant seeds, as in castor bean seed [2], peanut [3],

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and Barbados nut [4, 5], it is apparently inactive in vivo but highly active in vitro. In other words, as long as the seed is in the intact state, the enzyme will remain inactive, but any slight change in the seed or storage conditions will probably initiate activity [6].

Oilseed lipases have great potential for commercial exploitation as industrial enzymes, especially those oilseeds that are presently considered underutilized [6]. They have attracted much interest because of their many potential applications in synthetic reactions. The applications of lipases in industry include the synthesis of food ingredients, oil chemistry, food, organic synthesis, paper manufacturing, biosurfactants, cosmetics, pharmaceuticals, biodiesel and the use as additives in detergents [7–9].

However, our knowledge in the area of plant lipases is still very limited, especially when compared with information on mammalian and bacterial lipases, and although lipases used in industry have been obtained from microbes, the unique substrate specificity of plant lipases, not found in microbes or mammalian systems, may be of special value in industrial utilization [8,10].

Pachira aquatica is a tree belonging to the Bombacaceae family, found in a large area, from Southern Mexico to Northeastern Brazil. Its seeds are sometimes consumed raw or as roasted beans having a chestnut flavor, being considered a delicacy. Furthermore, its young leaves and flowers are cooked and used as vegetables [11].

A preliminary characterization showed the presence of a lipase, and accordingly, this study intended to carry on its purification and characterization.

Materials and Methods

Materials

The seeds were collected from trees of our Campus, at São José do Rio Preto, State of São Paulo. They were washed, peeled, and extracted by homogenization in a food processor with a solution containing: 3 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM sodium metabissulfite, and 50 mM Tris–HCl buffer (pH 8.0) at 25°C. A 0.3 ratio seed *w*/*v* extraction buffer was used, and the enzyme was recovered in the supernatant. The samples were clarified and concentrated by centrifugation at 9,000×*g* by 40 min at 4°C using a *Jouan* CR3i refrigerated centrifuge and stored until use in liquid nitrogen.

Purification

The purification procedure involved a zymogram method based on the protocol described by Fuciños et al. [12], with modifications. The SDS-PAGE was performed using 13% polyacrilamide slab gels with 0.1% sodium dodecylsulfate (SDS). For esterase detection, the gel was submerged in a solution containing α- and β-naphtyl acetate in the presence of Fast Blue [13]. The band was cut off, and the enzyme was allowed to diffuse in Tris–HCl buffer for 24 h. Purity of the enzyme was established as described by Laemmli [14]. For estimation of the relative molecular weight, we carried out 13% SDS-PAGE using molecular weight standards: aprotinin (6.5 kDa), alfa-lactalbumin (14.2 kDa), soybean trypsin inhibitor (20 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa), and albumin (66 kDa) purchased from Sigma. For protein detection, the gel was stained with Coomassie Brilliant Blue.

Assay of Lipase Activity

Lipolytic activity was estimated by a spectrophotometric assay method [15] using *p*-nitrophenyl palmitate (*p*-NPP) as substrate for this standard assay. The assay mixture contained 2.5 mM of the substrate, 2% of Triton X-100 as emulsifier, 50 mM Tris–HCl buffer pH 8.0 and 5 mg/ml of the pure enzyme. The assay mixture was incubated for 90 min at 37°C, and the reaction proceeded almost linearly in this time. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute at 37°C and pH 8.0.

As *p*-NPP is not stable at high pH values, assays of the pH effect were carried out by alkali titration using soybean oil as substrate as described by Saxena et al. [16] with some modifications. The assay mixture consisted of 50% soybean oil, 31.3% 30 mM Tris–HCl buffer pH 8.0, 10.15% Triton X-100, 0.002% of 10 mM $CaCl_2$, and 9.6% of the enzyme crude solution. Incubation was also performed for 90 min at 37°C. This assay was used also to determine the hydrolysis of natural oils.

The reaction was ended by adding of a mixture of acetone:ethanol (1:1), and the amount of released fatty acids was titrated with 50 mM KOH in the presence of phenolphthalein as indicator. One unit of enzyme activity was defined as the amount able to release 1 μmol of free fatty acids per minute and specific activities as micromole of product per minute per milligram protein, under the assay conditions. Protein concentration of the samples was estimated according to the dye binding assay method using bovine serum albumin as a standard [17].

Effect of Temperature

The effect of temperature on lipase activity was studied by carrying out the assays at different temperatures in the range of 25–60°C at pH 8.0 using 50 mM Tris–HCl buffer. Lipase thermostability was tested by preincubating the enzyme at varying temperatures ranging from 4 to 60°C for different time intervals, from 0 to 240 min.

pH Effect

The pH activity profile was studied by the alkali titration assay procedure described above in a pH range from 3.0 to 10.0 using different buffers at 50 mM concentration: sodium citrate (pH 3 to 5), Ada (pH 6 and 6.5), sodium phosphate (pH 7 and 7.5), Tris–HCl (pH 8 and 9), and glycine (pH 10).

Effect of Oxidizing, Reducing, and Chelating Agents on Lipase Activity

The effect of the chemicals was studied using a final concentration of 10 mM at 37°C and pH 8.0.

Effect of Solvents

The effect of several solvents was analyzed with 50% of solvent using the standard assay.

Kinetic Study

The effect of substrate concentration (*p*-nitrophenyl acetate) on the reaction rate was assayed at 37°C pH 7.5 (due instability of substrate) with 50 mM Hepes buffer measuring

the absorbance at 410 nm using a Varian Cary 100 Spectrophotometer. The limiting rate V_{max} and the Michaelis concentration, K_m, were calculated by nonlinear regression using GnuPlot 4.0 [18].

Substrate Specificity

Substrate specificity of the enzyme was determined at 37°C using 50 mM Hepes buffer pH 7.5 (due instability any substrates) and the assay method described above. Several nitrophenylesters were analyzed: *p*-nitrophenyl acetate (2:0), butyrate (4:0), caprate (10:0), myristate (14:0), palmitate (16:0), and stearate (18:0), as well as *O*-nitrophenyl palmitate. The specificity was also tested using soybean, olive, corn, sunflower, cotton, and rapeseed oils by titration with KOH as described previously.

Effect of Different Ions and Salt Concentration

The assays were performed using standard method in the presence of different ions: $CaCl_2$, $CoCl_2$, KCl, $HgCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, $AlCl_3$, and $NaCl_2$. We also tested the effect of salt concentration for LiCl, NaCl, and KCl.

Results and Discussion

The results obtained in the present study show lipase activity in ungerminated (dormant) seeds of the *P. aquatica*.

Purification

The lipase was purified 9.6-fold (Table 1). The purity of the lipase was checked by SDS-PAGE, and the enzyme had a molecular weight of approximately 55 kDa stained with Coomassie blue, but silver staining also showed the same results. (Fig. 1).

Effect of Temperature and pH

The activity reached a maximum at 40°C when tested with p-NPP and soybean oil (Fig. 2a) for a 90-min incubation period. The thermal stability was studied from 4 to 60°C (Fig. 2b). The data indicates that enzyme had appreciable stability during prolonged incubation (3 h) from 4 to 50°C. The enzyme exhibited maximum stability at 4°C and decreased at other temperatures.

The pH optimum (Fig. 3) was found at pH .0. This finding agrees with results reported for other plant lipases that display pH optima at alkaline values [3, 8, 19, 20] and is also consistent with the pH optima reported for lipases from other sources [1, 16, 21, 22].

Table 1 Purification of the lipase from *P. aquatica*.

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	2.5	168.5	2.625	64.2	100	1.0
electrophoresis	0.5	11.6	0.019	614.3	7	9.6

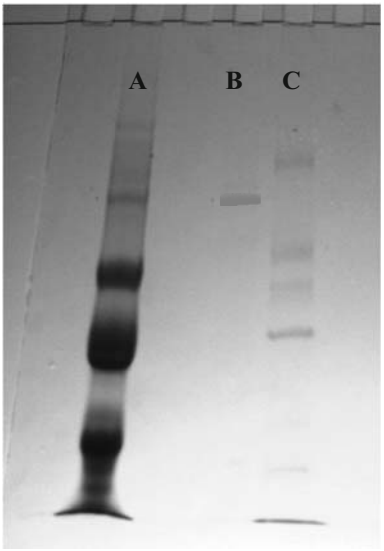

Fig. 1 SDS-PAGE electrophoresis pattern of the purified lipase from *P. aquatica* stained with Coomassie Brilliant Blue R-250. *Lane A*: crude sample, *lane B*: purified lipase, *lane C*: molecular mass markers

Effect of Oxidizing, Reducing, and Chelating Agents on Lipase Activity

The activity of the lipase from *P. aquatica* decreased in the presence of all the tested chemicals (Table 2). Only with oxidized glutathione, we observed the lowest inhibition; the enzyme retained 98% of the relative activity. Almost 30% of the activity is lost in the presence of 10 mM EDTA, and the effect was more prominent in the presence of reduced glutathione, DTT, and β-mercaptoethanol. This inhibition suggests that the lipase requires one or more intact disulfide bonds for maintaining its native conformation, as postulated for other lipases [23].

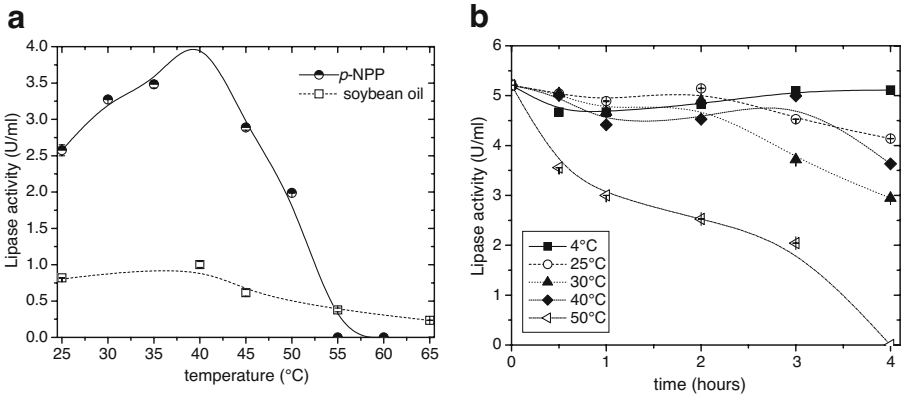

Fig. 2 **a** Effect of temperature on the activity of the lipase from *P. aquatica*. Activity was measured with *p*-NPP (*empty squares*) and soybean oil (*filled circles*) at pH 8.0. **b** Temperature stability measured using *p*-NPP as substrate. The experiments were performed in triplicate

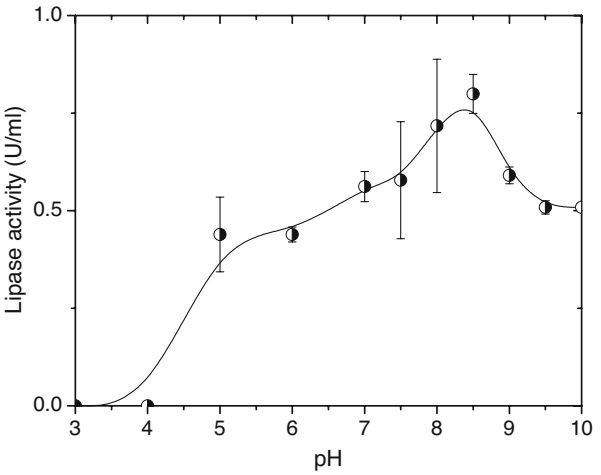

Fig. 3 Effect of pH on lipase activity, measured at 37°C. The experiments were performed in triplicate

EDTA inhibition of the enzyme activity could be attributed to its chelating ability. It will naturally perform what is known as the "chelation process" of the system and thereby disrupt the formation of the enzyme–substrate complex [6].

Effect of Solvents

Lipases are known for their ability to work in aqueous and in organic solvents. Table 3 shows the relative lipolytic activity towards *p*-nitrophenyl palmitate in the presence of 50% (*v*/*v*) organic solvents. The activity in the presence of methanol and glycerol was higher. Heller and Mozes [24] also observed increase of activity in the presence of methanol to phospholipase from peanut. The activities were lowered by the addition of ethanol and butanol, in agreement with findings reported by Kambourova et al. [22] and Takeda et al. [25] for lipases from *Bacillus stearothermophilus* and *Burkholderia cepacia*, respectively. The results from the present study indicate that *P. aquatica* lipase could be suitable for organic synthesis, as an important criterion in the selection of biocatalyst is its ability to be adequately stable under process conditions [26].

Table 2 Effects of chemical agents (10 mM) on lipase activity.

Agents	Relative activity (%)
Control	100.0
Ammonium persulfate	32.4
Potassium iodide	53.6
Glutathione (reduced)	65.7
Glutathione (oxidized)	98.
Ascorbic acid	44.0
DTT	1.0
β-mercaptoethanol	1.3
Sodium citrate	64.6
EDTA	70.9

The experiments were performed in triplicate.

Table 3 Relative activity of the purified lipase in organic solvents (50% *v/v*).

Solvents	Relative activity (%)
Control	100.0
Methanol	130.0
Ethanol	82.5
Glycerol	116.2
DMSO	93.6
Hexane	99.0
Butanol	82.0
Acetone	86.6

The experiments were performed in triplicate.

Kinetic Studies

The values of K_m and V_{max} of the purified lipase from *P. aquatica,* using *p*-NPP were 1.65± 0.026 mM and 37.3±0.024 μmol/mg min, respectively. The K_m value is higher than that found by Mohamed et al. [8] for the esterase III from *Avenua fatua* (0.38 mM) and higher than that described by Staubmann et al. [19] for esterases from *Jatropha curcas* (0.02 and 0.07 mM). The value of V_{max} is higher than the values reported for the lipase from *M. cephalus* (20 μmol/mgmin) and for esterases from *J. curcas* (0.26 and 0.24 μmol/mg min) [19, 27].

Substrate Specificity

The enzyme was able to hydrolyze both soluble and insoluble emulsified substrates. The hydrolysis rate for nitrophenylesters (Fig. 4a) were as follows: myristate > caprate > palmitate > stearate > *o*-palmitate > butyrate > acetate. The enzyme preferentially hydrolyzed long-chain fatty acid esters and showed the highest activity for myristate esters as found by Takeda et al. [25] for cholesterol esterase of the bacterium *B. cepacia.* The enzyme showed activity in different oils, but exhibited the highest one for olive oil (Fig. 4b)

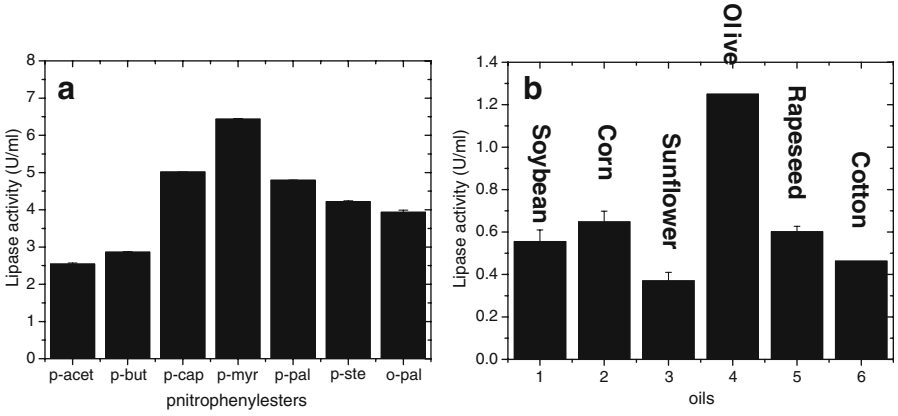

Fig. 4 Lipase activity on different substrates (**a**) using nitrophenylesters, measuring at 37°C and pH 7.5. **b** enzyme activity on different oils at 37°C and pH 8.0. The experiments were performed in triplicate

Table 4 Effects of various cations at 10 mM on the lipolytic activity.

Cation	Relative activity (%)
None	100.0
Ca	222.0
Mg	208.0
Li	92.0
Co	68.0
Na	85.0
K	77.0
Zn	38.0
Al	15.0
Mn	13.5
Hg	9.4

The experiments were performed in triplicate.

followed by corn and rapeseed oil. This enzyme is a lipase that displays some esterase activity as well, as noted by Gilbert et al. [28] and Kambourova et al. [22] for bacterial lipases from *Pseudomonas aeruginosa* and *Bacillus stearothermophilus*, respectively.

This comparison is, however, tentative, due to different physical state of the substrates and interfacial parameter which have been shown to influence the rate of lipase hydrolysis [27].

Effect of Different Cations and Salt Concentration

Table 4 shows the effect of different ions on lipase activity. Mg^{++} and Ca^{++} ions doubled the lipolytic activity, whereas Hg^{++}, Mn^{++}, Zn^{++}, and Al^{+++} caused significant inhibition. This inhibition was also demonstrated by esterase from *Jatropha curcas* [19]. Similarly, Aisaka and Terada [29], Sanz and Olias [30], and Saxena et al. [15] have reported for other lipases from Rhizopus *japonicus*, lupin seed, and *Aspergillus carneus*, respectively, activation effects for Ca^{++} and Mg^{++}. Some enzymes were activated only by Ca^{++}, such as the lipase from *A. fatua* esterases [8], *J. curcas* L [4], castor bean acid lipase [31], and

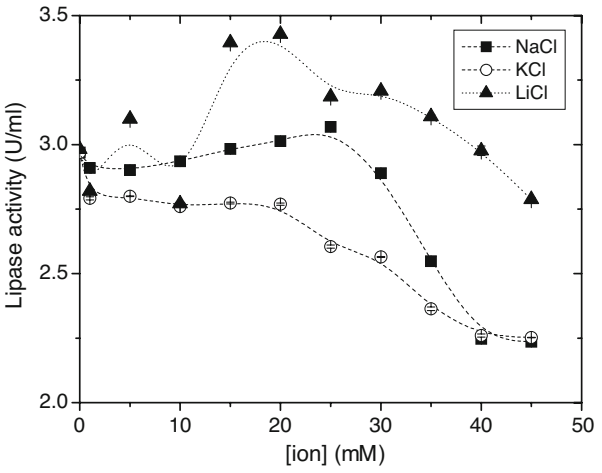

Fig. 5 Effect of three cations: Na, K and Li on lipase activity, determined at 37°C and pH 8.0. The experiments were performed in triplicate

also *Pentaclethra macrophylla* lipase [6]. In general, lipases are strongly inhibited by Hg^{2+} (a thiol group inhibitor). This is likely due to the proximity of the SH group to the catalytic and interfacial binding site but spatially remote from catalytic site, this may have induced the marked loss of activity [27, 32, 33]. The catalytic triad of lipases has been recognized to consist of Ser, His and Glu or Asp [34, 35], thus the bulky Hg^{2+} group might cause steric interference to the approach of the substrate to the active site.

Figure 5 shows the effect of cation concentration in the lipolytic activity. In agreement with our data, Yu et al. [36] observed that Li^{+} stimulated a lipase from *Candida rugosa* more than Na^{+} and K^{+}. It is not clear why Li, Na, and K have different effects on lipase activity. One reason suggested by Yu et al. [36] is that lithium is chemically more efficient to facilitate the contact between the substrates and the lipase.

Ions at lower concentrations have a stimulant effect, probably because the salt exerts a screening effect and disperse the enzyme molecules. When salt concentration increases, the activity would decrease because it could cover the lipase surface and prevent substrate binding. Rather, they provide a driving force for substrate binding and catalysis by lowering energy barriers in the ground and/or transition states and enhance enzyme activity through conformational transitions triggered upon binding to a site where the ion makes no direct contact with substrate [37].

The lipase from *P. aquatica* presented in this study has some interesting characteristics such as wide substrate specificity and could be used for different purposes in the oil industry.

References

1. Salah, R. B., Mosbah, H., Fendri, A., Gargouri, A., Gargouri, Y., & Mejdoub, H. (2006). *FEMS Microbiology Letters*, *260*, 241–248.
2. Huang, A. H. C. (1984). In B. Borgstrom, & H. L. Brockman (Eds.) *Lipase: Plant lipases* (pp. 419–422). Amsterdam: Elsevier Publishers.
3. Sanders, T. H., & Pattee, H. E. (1975). *Lipids*, *10*(1), 50–54.
4. Abigor, R. D., Uadia, P. O., Foglia, T. A., Haas, M. J., Scott, K., & Savary, B. J. (2002). *Journal of the American Oil Chemists Society*, *79*(11), 1123–1126.
5. Haas, M. J., Cichowicz, D. J., & Bailey, D. G. (1992). *Lipids*, *27*, 571–576.
6. Enujiugha, V. N., Thani, F. A., Sann, T. M., & Abigor, R. D. (2004). *Food Chemistry*, *88*, 405–410.
7. Rubin, B., & Dennis, E. A. (1997). *In methods enzymology: Lipases*. New York: Academic Press.
8. Mohamed, M. A., Mohamed, T. M., Mohamed, S. A., & Fahmy, A. S. (2000). *Bioresource Technology*, *73*, 227–234.
9. Sharma, R., Soni, S. K., Vohra, R. M., Gupta, L. K., & Gupta, J. K. (2002). *Process Biochemistry*, *37*, 1075–1084.
10. Huang, A. H. C., Lin, Y. H., & Wang, S. (1988). *Journal of the American Oil Chemists Society*, *65*, 897–899.
11. Lorenzi, H. (1992). *Árvores Brasileiras: Manual de identificação e cultivo de plantas arbóreas nativas do Brasil*. São Paulo: Editora Plantarum LTD.
12. Fuciños, P., Abadín, C. M., Sanromán, A., Longo, M. A., Pastrana, L., & Rúa, M. L. (2005). *Journal of Biotechnology*, *117*, 233–241.
13. Beguin, P. (1983). *Analytical Biochemistry*, *131*, 333–336.
14. Laemmli, U. K. (1970). *Nature*, *227*, 680–685.
15. Chang, C. R., Chou, S. J., & Shaw, J. F. (1994). *Biotechnology and Applied Biochemistry*, *19*, 93–97.
16. Saxena, R. K., Davidson, W. S., Sheoran, A., & Giri, B. (2003). *Process Biochemistry*, *39*, 239–247.
17. Bradford, M. (1976). *Analytical Biochemistry*, *72*, 248–254.
18. Williams, T., & Kelley, C. (2004). GnuPlot for Windows 4.0.
19. Staubmann, R., Ncube, I., Gübitz, G. M., Steiner, W., & Read, J. S. (1999). *Journal of Biotechnology*, *75*, 117–126.
20. Bhardwaj, K., Raju, A., & Rajasekharan, R. (2001). *Plant Physiology*, *127*, 1728–1738.

21. Ghanem, E. H., Al-Sayed, H. A., & Saleh, K. M. (2000). *World Journal of Microbiology and Biotechnology*, *16*, 459–464.
22. Kambourova, M., Kirilova, N., Mandeva, R., & Derekova, A. (2003). *Journal of Molecular Catalysis B: Enzymatic*, *22*, 307–313.
23. Nawani, N., Khurana, J., & Kaur, J. (2006). *Molecular and Cellular Biochemistry*, *209*, 17–22.
24. Heller, M., & Mozes, N. M. (1981). *Methods Enzymology*, *35*, 226–232.
25. Takeda, Y., Aono, R., & Doukyu, N. (2006). *Extremophiles*, *10*, 269–277.
26. Faber, K., & Franssen, M. C. R. (1993). *Trends in Biotechnology*, *11*, 461–470.
27. Aryee, A., Simpson, B. K., & Villalonga, R. (2007). *Enzyme and Microbial Technology*, *40*, 394–402.
28. Gilbert, E. J., Cornish, A., & Jones, C. W. (1991). *Journal of General Microbiology*, *137*, 2223.
29. Aisaka, K., & Terada, O. (1981). *Journal of Biochemistry*, *89*, 817–822.
30. Sanz, L. C., & Olias, J. M. (1990). *Food Chemistry*, *37*, 221–228.
31. Eastmond, P. J. (2004). *The Journal of Biological Chemistry*, *279*(44), 45540–45545.
32. Raso, B. A., & Hultin, H. (1988). *Comparative Biochemistry and Physiology*, *89*(B), 671–677.
33. Verger, R., Srada, L., & Desnulle, P. (1971). *Biochimica et Biophysica Acta*, *242*, 580–592.
34. Guidoni, A. A., Bendouka, F., De Caro, J. D., & Rovery, M. (1981). *Biochimica Et Biophysica Acta*, *660*, 148–150.
35. Jin, W., Broedl, U. C., Monajemi, H., Gllick, J. M., & Rader, D. J. (2002). *Genomics*, *80*, 268–273.
36. Yu, H. W., Chen, H., Yang, Y. Y., & Ching, C. B. (2005). *Journal of Molecular Catalysis B: Enzymatic*, *35*, 28–32.
37. Di Cera, E. (2006). *The Journal of Biological Chemistry*, *281*(3), 1305–1308.