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Production of Lipase by a Newly Isolated *Bacillus coagulans* Under Solid-State Fermentation Using Melon Wastes

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Abstract

An extracellular lipase was produced by *Bacillus coagulans* by solid-state fermentation. Solid waste from melon was used as the basic nutrient source and was supplemented with olive oil. The highest lipase production (78,069 U/g) was achieved after 24 h of cultivation with 1% olive oil enrichment. Enzyme had an optimal activity at 37°C and pH 7.0, and sodium dodecyl sulfate increased lipase activity. NH_4NO_3 increased enzyme production, whereas organic nitrogen had no effect. The effect of the type of carbon sources on lipolytic enzyme production was also studied. The best results were obtained with starch and maltose (148,932 and 141,629 U/g, respectively), whereas a rather low enzyme activity was found in cultures grown on glucose and galactose (approx 118,769 and 123,622 U/g, respectively). Enzyme was inhibited with Mn^{+2} and Ni^{+2} by 68 and 74%, respectively. By contrast, Ca^{+2} enhanced enzyme production by 5%.

Index Entries: Solid-state fermentation; *Bacillus*; lipase production.

Introduction

Lipases (EC 3.1.1.3), which catalyze the hydrolysis of triglycerides to fatty acids and glycerol, have been widely used in the modification of fats and oils *(1)*. Interest in these enzymes has increased markedly over the last decades, because they have found diverse applications as medicines (digestive enzymes), clinical reagents (glyceride-hydrolyzing enzymes),

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and cleaning agents (detergent additives) *(2,3)*. Most of the research has been concentrated on extracellular lipases produced by a wide variety of microorganisms including bacteria, fungi, yeast, and actinomyces *(4–8)*.

Many reports of solid-state fermentation (SSF) systems have recently been published supporting the application of SSF in agricultural byproducts and in the production of fine chemicals and enzymes. SSF processes are therefore of special economic interest for countries with an abundance of biomass and agroindustrial residues, because these can be used as cheap raw materials *(2,9)*. Not only does the application of agroindustrial residues in bioprocesses provide alternative substrates, but it also helps solve pollution problems. Biotechnological processes, especially the SSF technique, have contributed enormously to such utilization *(10)*.

The aim of the present study was to devise an SSF process using a novel substrate with a new *Bacillus coagulans* strain isolated from hot-springs water and identified for its capacity to grow rapidly on a solid support and produce large quantities of extracellular lipase.

Materials and Methods

Microorganism

B. coagulans isolated from hot-springs water in Diyarbakır, Turkey was used as the biologic material. It was grown on nutrient broth (NB) medium at 37°C for 24 h for preparation of the inoculum. Two hundred fifty microliters of the growth was transferred to NB medium containing 1% olive oil and incubated for 48 h.

Lipase Production by SSF

Different substrates such as wheat bran, banana waste, melon waste, watermelon waste, lentil husk, and rice husk were tested for lipase production by SSF. Samples (30% [w/v]) of substrates were taken in a series of 250mL Erlenmeyer flasks; moistened with 25 mL of 0.1 *M* Tris-HCl buffer (pH 7.0) and 1% olive oil; and then sterilized at 121°C, 1.05 kg/cm^2 for 15 min. After cooling, the flasks were inoculated with a 15% inoculum concentration (3.5×10^8 spores/mL) and then incubated at 37°C for 5 d. Samples were taken at 24-h intervals and assayed for enzyme activity.

Extraction of Enzyme

At the end of the fermentation period, bacterial bran was extracted with 20 mL of 50 m*M* Tris-HCl buffer (pH 7.0). The slurry was squeezed through a damp cheesecloth. The filtrate was centrifuged at 5000*g* for 10 min, and the supernatant was used as the enzyme source.

Enzyme Assay

A spectrophotometric method was used for lipase activity using *p*-nitrophenylpalmitate (*p*-NPP) as the substrate. The reaction mix-

ture, containing 100 μL of 50 m*M* Tris-HCl (pH 7.0), 150 μL of substrate solution (1 m*M p*-NPP containing 1% Triton X-100), and 350 μL of H_2O, was kept at 37°C and the reaction was started by adding enzyme solution (100 μL). After incubation at 37°C for 10 min, the reaction was stopped by adding 1 mL of 2% sodium dodecyl sulfate (SDS) solution. The absorbance at 420 nm was determined. One unit of lipase activity was defined as the amount of enzyme liberating 1 μmol of p-nitrophenol/min *(11)*.

Effect of pH and Temperature on Lipase Activity and Stability

The optimum pH of enzyme activity was determined by measuring at pH 4.0–8.0 in various buffers. The effect of pH on lipase stability was studied by measuring residual activity after 1 h of incubation at pH ranging from 5.0 to 9.5.

The optimum temperature was determined by measuring the rate of the reaction at temperatures ranging from 30 to 60°C under standard assay conditions. To study thermal stability, enzyme was incubated at 30–80°C for 1 h and residual activity was determined.

Optimization Studies

Consecutive optimization studies were carried out by varying the substrate and the incubation time from 24 to 120 h. The effects of the addition of different oils (1% [v/v] of sunflower oil, corn oil, soybean oil, olive oil, and tributyrin), oil volume (1, 2, 3, 4, 5, 6, and 7%), various nitrogen and carbon sources (2% [w/v]), surfactants (1% [v/v] of SDS, Triton X-100, polyethylene glycol [PEG], and gum arabic), and metal ions (0.1% [w/v] of $CaCl_2$, $CuSO_4$, $MgSO_4$, $NiSO_4$, $ZnSO_4$, $MnSO_4$, and $FeSO_4$) were studied for optimal lipase production.

Results and Discussion

Bacillus species are good lipase producers and are preferred in the food industry for enzyme production, owing to their nonpathogenicity *(12)*. Different agricultural byproducts including wheat bran, banana waste, lentil husk, rice husk, melon waste, and watermelon waste were evaluated to select the best supporter of lipase production by *B. coagulans* (Fig. 1). Among the various substrates tested, melon waste was found to be the best substrate for lipase production. Further studies were carried out using melon waste as the substrate. This is the first report on lipase production using melon waste as the substrate in SSF.

Figure 2 presents the time course of lipase production by *B. coagulans*. Lipase production reached a peak of 78,069 U/g at 24 h, and a decrease in enzyme production was observed at less than and greater than 24 h of growth. This might be owing to denaturations and/or decomposition of lipase as a result of interactions with other compounds in the fermented medium.

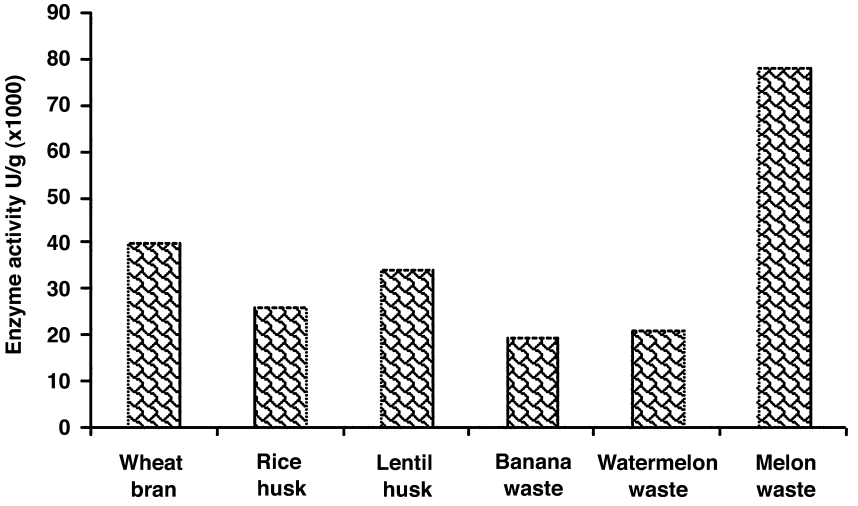

Fig. 1. Effect of different agricultural wastes on production of lipase by *B. coagulans*.

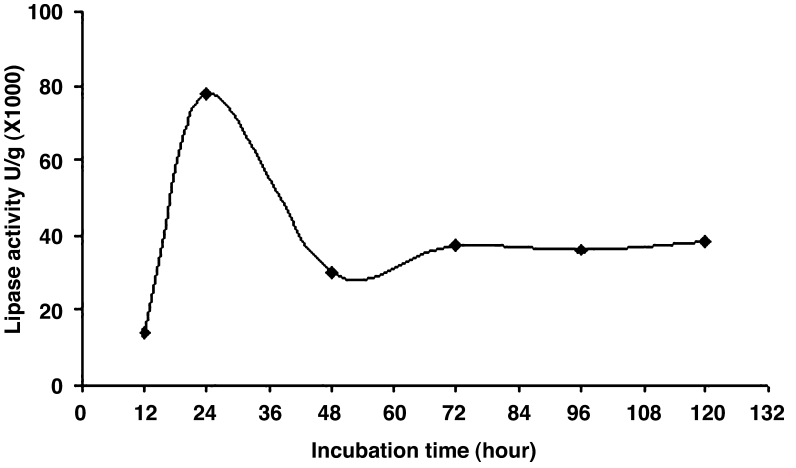

Fig. 2. Effect of incubation time on lipase production in SSF.

Lipase production is influenced by pH, temperature, and medium composition. The protein nature of enzymes means that pH will affect the ionization state of the amino acids, which dictates the primary and secondary structure of the enzyme and, hence, controls its overall activity. A change in pH will have a progressive effect on the structure of the protein and the enzyme activity *(13)*. Lipase assay was performed from pH 4.0 to 8.0 using a variety of buffers at 50 m*M*. The optimum activity of lipase was observed at pH 7.0. In pH stability experiments, more than 72% activity was recovered after 1 h of incubation of lipase at pH ranging from 5.0 to 8.0. At pH 9.5, 43% activity was lost under the same conditions (Fig. 3).

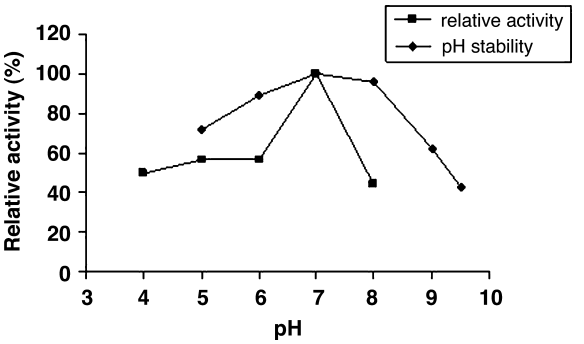

Fig. 3. Effect of pH on activity of *B. coagulans* lipase and enzyme stability.

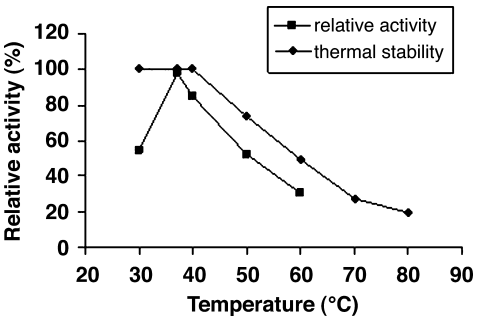

Fig. 4. Effect of temperature on lipase activity and enzyme stability.

Isolates of *Bacillus* sp. have been found to produce lipolytic enzymes under alkaline conditions *(14)*. Lipase from Bacillus subtilis and *Bacillus licheniformis* have been of particular interest because they exhibit optimal activity and stability at extreme alkaline pH values greater than 9.5 *(15)*. These enzymes, however, are thermolabile. Another lipase produced by a *Bacillus* sp. RSJ-1 has shown maximum activity at pH 8.0–9.0 *(13)*. These results are in contrast to those of lipase from the *Bacillus* that we tested, *B. coagulans*, which is thermotolerant but displays maximum activity at moderate alkaline pH values (pH 7.0). Rua et al. *(8)* and Lee et al. *(16)* found similar results from *Bacillus thermoacetunulatus* and *Bacillus thermoleovorans*, respectively.

Lipase activity was determined at different temperatures under standard assay conditions; Figure 4 presents the results. The enzyme exhibited maximum activity at 37°C, and activity decreased above and below the optimum temperature. The results of the thermostability studies presented in Fig. 4 reveal that the enzyme exhibited 49% of its original activity when incubated at 60°C for 1 h, that the enzyme was inactivated at 70°C, and that activity was lost at 80°C after 1 h of incubation.

Table 1
Effects of Different Oils on Lipase Production

Oil (1%)	Lipase activity (U/g)
Olive oil	78,069
Soybean oil	45,526
Sunflower oil	52,437
Corn oil	19,249
Tributyrin	52,208

Table 2
Effects of Olive Oil Concentration in Medium on Lipase Production

Concentration of olive oil (%)	Lipase activity (U/g)
1.0	78,069
2.0	140,487
3.0	97,424
4.0	49,178
5.0	133,061
6.0	39,430
7.0	19,390

SSF cultures of *B. coagulans* were carried out using a complex medium supplemented with several lipidic compounds: olive oil, sunflower oil, corn oil, soybean oil, and tributyrin. These materials have been reported to induce lipase secretion by some microorganisms, although their effect is variable and, therefore, difficult to generalize *(4,17–19)*. All oils tested supported enzyme production (Table 1). However, the highest enzyme activity was observed in cultures containing olive oil (1%). Next, various olive oil concentrations were assayed in order to determine the influence of this variable on lipase production by *B. coagulans*. Table 2 provides average maximum values for lipolytic enzyme production. Enzyme activity appeared to increase with 2% olive oil. Therefore, this olive oil concentration was chosen as an economically optimum value for subsequent experiments. Above this concentration, we observed that the enzyme production decreased, because of the viscosity of the medium, which prevented aeration.

The addition of surfactants to the culture medium has been shown to enhance the secretion of lipolytic enzymes by a number of microorganisms. This may be attributed to the occurrence of changes in cell wall permeability or to the effects of SDS on cell-bound enzyme. Although Tween-80 appears to be the most commonly used surfactant in lipase/esterase production cultures, some compounds have been assayed (e.g., Triton, SDS, or PEG) *(17,20–22)*. Therefore, several surfactants (SDS, Triton X-100, PEG, and gum arabic) were added to the culture medium in order to assess their

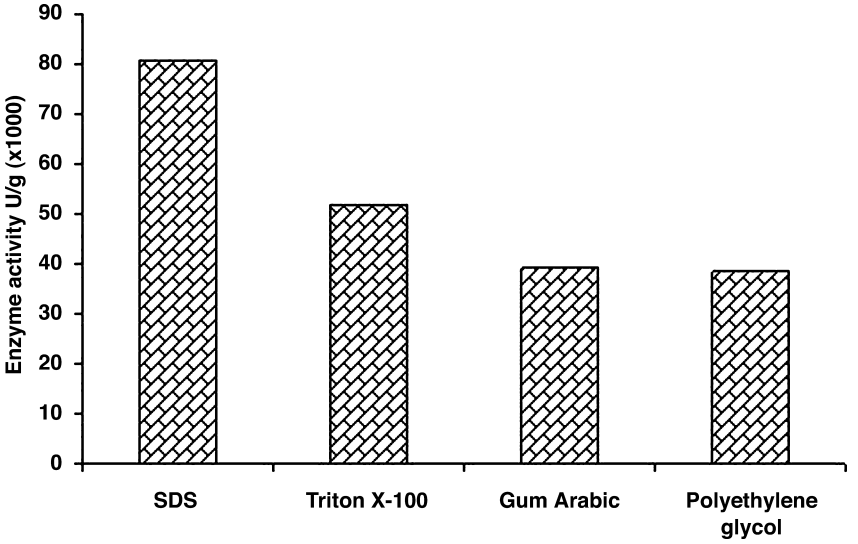

Fig. 5. Effect of surfactants on lipase production.

influence on lipolytic enzyme production by *B. coagulans*. In the studied conditions, no significant increase was observed expect when SDS was added, and lipase production rate was similar with gum arabic and PEG (Fig. 5).

Nitrogen sources, including organic and inorganic nitrogen compounds, play an important role in the synthesis of enzyme. Table 3 presents the results of the influence of inorganic and organic nitrogen sources on lipase production. Among the inorganic nitrogen sources used, NH_4NO_3 was the optimal one, increasing lipase production. The addition of different organic nitrogen sources to the substrate did not influence lipase production (Table 3). The addition of different organic and inorganic nitrogen sources to the substrate did not influence lipase production with *Aspergillus niger*. The addition of beef extract to gingelly oil cake enhanced lipase production during optimization studies *(23)*. In the case of *Candida rugosa*, the addition of urea to rice bran enhanced lipase production *(24)*.

Carbon is the main component of cells and some sugars have been used as carbon sources and inducers for lipase fermentations *(4,25)*. A range of different carbon sources has been reported to support both growth of lipolytic enzyme producers and lipase/esterase production *(19,26,27)*. Our studies on carbohydrate supplementation indicated that the addition of starch and maltose resulted in a slight increase in lipase production (Table 3). Glucose and fructose have been reported to favor lipase production by *Issatchenksa orientalis (4)*.

Metal ions and salts are important for enzyme activity. A number of enzymes require the presence of metal ions, such as calcium ions, for the maintenance of their stable and active structures. These ions are bound

Table 3
Effects of Nitrogen (Organic and Inorganic) and Carbon Sources on Lipase Production

Nitrogen and carbon source (2% [w/v])	Lipase activity (U/g)
NH_4Cl	131,607
NH_4SO_4	109,065
NH_4NO_3	161,898
$NH_4H_3PO_4$	111,822
NaH_2PO_4	80,921
Na_2HPO_4	82,245
Urea	69,155
Casoaminoacid	99,906
Peptone	81,532
Beef extract	94,013
Yeast extract	97,404
Bacto liver	106,732
Sucrose	138,914
Starch	148,932
Glucose	118,769
Galactose	123,622
Maltose	141,629

strongly to specific binding sites on the surface of the molecules. The binding sites are usually constructed from negatively charged carboxylate side-chain groups of aspartyl and glutamyl residues, brought together by folding of the polypeptide chain. Dissociation constants for binding are low (of the order of 10^{-3} to 10^{-6} *M*) in the case of $E + Ca^{+2} \leftrightarrow E - Ca^{+2}$, representing very strong binding and also emphasizing that the effects took place at low calcium ion concentrations. As can be seen from this equation, the situation is similar to that of enzyme-substrate complexes. The polypeptide chain is crosslinked by the metal ion bridge, and the enzyme-calcium ion complex therefore should be more rigid and more stable. The bridging by metal ions in this way is compared with that brought about by the formation of disulfide. In the absence of calcium ions, the binding site would represent a high local concentration of negative charges. The tendency of these groups to move apart to reduce the repulsive electrostatic interactions would contribute to the relative instability of the folded protein *(13,28)*. In our study, 0.1% $CaCl_2 \cdot H_2O$ enhanced enzyme activity by 5%, and it was strongly inhibited to 68 and 74% by Mn^{+2} and Ni^{+2}, respectively (Table 4).

Based on the present results, it can be concluded that this strain of *B. coagulans* is able to produce lipase in SSF having melon waste as substrate. High lipase titers could be obtained using an abundant and cheap raw material. Short fermentation time (24 h) makes this fermentation system a promising one in terms of lipase productivity.

Table 4
Effect of Metal Ions Added on Lipase Production

Metal ion	Relative activity (% of control)
Calcium chloride	105
Cupric sulfate	71
Magnesium sulfate	76
Manganese sulfate	32
Zinc sulfate	71
Ferric sulfate	56
Nickel sulfate	26

Acknowledgment

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References

1. Tan, T., Zhang, M., Wang, B., Ying, C., and Deng, L. (2003), *Process Biochem.* **39,** 459–465.
2. Gombert, A. K., Pinto, A. L., Castilho, L. R., and Freire, D. M. G. (1999), *Process Biochem.* **35,** 85–99.
3. Sugiura, M. (1984), in *Lipases,* Brogström, B. and H. L., eds., Elsevier, Amsterdam, pp. 505–523.
4. Costas, M., Devie, F. J., and Longo, M. A. (2004), *Process Biochem.* **39,** 2109–2114.
5. Rapp, P. Backhaus, S. (1992), *Enzyme Microb. Technol.* **14,** 938–943.
6. Oh, B. C., Kim, H. K., Lee, J. K., Kang, S. C., and Oh, T. K. (1999), *FEMS Microbiol. Lett.* **179,** 385–392.
7. Essamri, M., Deyris, V., and Comeau, L. (1998), *J. Biotechnol.* **60,** 97–103.
8. Rua, M. L., Schmidt-Dannert, C., Wahl, S., Sparauer, A., and Schmis, R. D. (1997), *J. Biotechnol.* **56,** 89–102.
9. Pandey, A. (2003), *Biochem. Eng. J.* **13,** 81–84.
10. Soccol, C. R. and Vandenberghe, L. P. S. (2003), *Biochem. Eng. J.* **13,** 205–218.
11. Imamura, S. K. and Kituara, S. (2000), *J. Biochem.* **127,** 419–425.
12. Joyce, J. and Muraleedhara, K. (1999), *Indian J. Exp. Biol.* **37,** 1231–1217.
13. Sharma, R., Soni, S. K., Vohra, R. M., Gupta, L. K., and Gupta, J. K. (2002), *Process Biochem.* **37,** 1075–1084.
14. Lindsay, D., Brozel, V. S., Mostert, J. F., and von Holy, A. (2000), *Int. J. Food Microbiol.* **54,** 49–62.
15. Nthangeni, M. B., Patterton, H. G., van Tonder, A., Verger, W. P., and Litthauer, D. (2001), *Enzyme Microb. Technol.* **28,** 705–712.
16. Lee, D. W., Koh, Y. S., Kim, K. J., et al. (1999), *FEMS Microbiol. Lett.* **179 (2),** 393–400.
17. Marek, A. and Bednarski, W. (1996), *Biotechnol. Lett.* **18,** 1155–1160.
18. Maia, M. M. M., Heasley, A., Camargo de Morais, M. M., Melo, E. H. M., Morais, M. A. Jr., and Ledingham, W. M. (2001), *Bioresour. Technol.* **76,** 23–27.
19. Choi, Y. J. and Lee, B. H. (2001), *Bioprocess Biosyst. Eng.* **24,** 59–63.
20. Espinosa, E., Sanchez, S., and Farres, A. (1990), *Biotechnol. Lett.* **12,** 209–214.
21. Muralidhar, R. V., Chirumamila, R. R., Marchant, R., and Nigam, P. (2001), *Biochem. Eng. J.* **9,** 17–23.
22. Ferrer, P. and Sola, C. (1992), *Appl. Microbiol. Biotechnology* **37,** 737–741.
23. Kamini, N. R., Mala, J. G. S., and Puvanakrishnan, R. (1998), *Process Biochem.* **33,** 505–511.

24. Verkata, R., Kunthala, P., and Lakshmanan, C. M. (1993), *Process Biochem.* **28,** 391–395.
25. Mahadik, N. D., Bastawde, K. B., Puntambekar, U. S., Khire, J. M., and Gokhale, D. V. (2004), *Process Biochem.* **39,** 2031–2034.
26. Dalmau, E., Mostesinos, J. L., Lotti, M., and Casas, C. (2000), *Enzyme Microb. Technol.* **26,** 657–663.
27. Conzo, G. and Revah, S. (1999), *Bioresour. Technol.* **70,** 173–180.
28. Gray, C. J. (1995), in Thermostability of Enzymes, Gupta, M. N., ed., Narosa, New Delhi, India, pp. 124–143.