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Optimization of growth conditions for levansucrase production by *bacillus licheniformis* in solid state fermentation

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ABSTRACT

Solid state fermentation processes involve the growth of microorganisms on a solid material in the absence or near absence of free-flowing water. Hence, the objective of the present study was the optimization of medium composition and cultural conditions for levansucrase enzyme production by *Bacillus licheniformis* under solid state fermentation using cheaper sources as agricultural residues. The enzyme production was affected by incubation periods, level of moisture content and carbon source supplementation. Maximum enzyme production of about 62.68 U/g of levansucrase was obtained under optimum conditions with an incubation period of 48 h, initial moisture content of 80% and in the presence of waste dates (6g) at 40ºC. The extraction of enzyme was found depending on different parameters such as the nature of extractant, the temperature and the pH, etc. Optimizing the SSF conditions increased the levansucrase production and this inexpensive enzyme production for such a potent and industrially valuable levansucrase is promising and of considerable commercial interest for biotechnological applications.

KEYWORDS

Bacillus licheniformis;
Levansucrase optimization;
Solid state fermentation;
Agroindustrial substrate.

INTRODUCTION

Levansucrases, which are fructosyltransferases (E.C.2.4.1.9) belonging to glycoside hydrolases family 68, catalyzes formation of fructooligosaccharides (FOS) and synthesis of β-(2–6) levan by transferring fructosyl group of non-activated sucrose into fructan chain[1]. FOS are extensively used in food industries because of their functional properties[2]. Levan has a lot of potential applications such as in food industry (as a low-caloric sweetener), cosmetics, pharmaceuticals, medicine as antitumor agent thanks to its physical properties[3], a hypocholesterolemic agent[4] and as an increasing agent of bifidobacteria population in the intestinal track[5].

Solid-state fermentation (SSF) produces product many-fold higher than that from submerged culture and has a relatively low energy requirement. Most of bacteria and fungi growing under SSF conditions capable of supplying global demand for various metabolites[6]. SSF by low cost materials is considered to be best way especially in developing countries.

There are many reports on the production of levansucrase by submerged fermentation (SmF)[7]. However, there are few reports on the production of levansucrase by solid state fermentation (SSF)[8]. SSF is defined as a process in which micro-organisms are grown on solid substrates in the absence or near ab-

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sence of free water and secrete the enzyme necessary for the degradation of the available substrate molecules in order to meet their optimum nutritional value. The production of enzymes by SSF has merit over SmF, with respect to the simplicity of the deployed technique, the similitude with natural conditions available for microorganisms, the product concentration, decrease of the growth of contaminants, increase of the production levels and substrates cost[9]. SSF appears as an interesting low cost alternative for the production of biomolecules because agro-industrial residues can be used as culture media which reduce production costs. Some of the used substrates include sugarcane bagasse, wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, soy hull, corncobs, banana waste, tea waste, cassava waste, apple pomace, etc.

Productivity was affected by the nature of solid substrate (SS), SS concentration, level of moisture content, pH of medium, incubation temperature and incubation period. In addition, the water activity in SSF is an important factor for microorganism growth and enzyme production.

In SSF, the products are formed at or near the surfaces of the SS with low moisture content, so it is necessary to select suitable solvents or solutions for leaching out the product from the bulk solid mass [10]. A common extractant is distilled or deionized water; other extractants have also been used to extract other enzymes such as using sodium chloride solution for protease extraction [11].

This paper reports the optimization of fermentation parameters for levansucrase production by *Bacillus. licheniformis* through SSF. Studies on the extraction of levansucrase from sawdust include the effect of some factors which influence the efficiency of leaching out the enzyme and their appropriateness to the leaching technique. Basic data, as the biochemical properties, is important to determine the best conditions for enzyme application in the industrial process. To best of our knowledge, SSF technique is yet to be explored for levansucrase production in SSF by using date as substrate and no studies were carried out on SSF production of levansucrase by *Bacillus licheniformis* because the strain was newly isolated [12].

MATERIALS AND METHODS

Microorganism and maintenance

Bacterium used throughout this work, *Bacillus licheniformis* was previously isolated and identified [12]. Bacterial strain was routinely grown on nutrient agar medium at 40°C and preserved at -80°C in 50% (v/v) glycerol.

Culture conditions

The selected bacterial strains were inoculated with 10% v/v (2×10^7 CFU/ml) in Luria-Bertani (L.B) broth medium (10.0g tryptone, 5.0g yeast extract and 5.0g NaCl in 1L of distilled water)[13] and incubated at 37°C for 18 h to get a standardized inoculum (0.5 OD at 600 nm with 3.5×10^5 CFU /ml).

Solid state fermentation

Experiments were conducted in 250 ml Erlenmeyer flasks containing 6g of the substrate moistened with 25 ml of sterile liquid nutrient medium containing Na_2HPO_4 3.5 g/l, NaH_2PO_4 0.8 g/l, $MgSO_4$ 0.2 g/l, $NaNO_3$ 3.5 g/l and yeast extract 5.0 g/l. The flasks were incubated at 40°C for 1 h and 160 rpm. Then the flasks were autoclaved for 20 min at 121°C and each flask was inoculated with 6 ml inoculum from 18 h old culture and incubated at 40°C for 48 h at static condition.

Optimization of solid state fermentation process

In a sequential order, the various physicochemical factors as substrate, moisture content and incubation time affecting the enzyme production were optimized for maximal enzyme production by using the solid substrate for which best levansucrase activity was observed.

Substrate

The enzyme production was studied with different solid substrates (sucrose, wheat bran, Product P, waste dates, starch, sawdust, almond, almond peels, melon peels, fig peels, watermelon peels, cactus peels) and five varieties of palm juice (*Beser*, *Thokkar*, *Ameri*, *Gasbi* and *Khalt*). The effect of solid substrate concentration on the enzyme production was measured at different concentrations of date (1, 2, 3, 4, 5, 6 and 7 g).

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The effect of culture conditions in the present study was carried out at different incubation periods (12, 24, 36, 48, 72 and 96 h) and different moisture content (10, 20, 40, 60, 70, 80 and 90%).

Extraction process

At the end of the fermentation process, the biomass was treated with 20 ml of distilled water and agitated thoroughly on a rotary shaker for 30 min. The whole contents were filtered and centrifuged at 8000 rpm for 15 min. The clear supernatant was stored at 4°C and used as crude enzymes for enzyme assay.

Effect of different solvents

The extraction of the enzyme from the fermented biomass was carried out with distilled water and inorganic salt solutions (potassium chloride, magnesium chloride, calcium chloride, and sodium chloride) at different concentrations (0.025, 0.05, 0.075, 0.1 and 0.125 %) and organic solvents (glycerol, methanol, ethanol and acetone) at a concentration of 5 %.

Effect of distilled water volume

An experiment was carried out to see the effect of solvent level (5, 10, 20, 30 and 40 ml) on enzyme extraction.

Effect of soaking pH

To study the effect of initial pH on the extraction process the pH was varied from 2.0 to 10.0.

Effect of soaking temperature

To study the effect of temperature on the extraction process, temperature was varied from 30°C to 60°C each at 10°C intervals.

Enzyme assay

Levansucrase assay was performed according to Yanase et *al.* method[14]. Decreasing amounts of sugars produced were measured by glucose oxidase kits. One unit of enzyme activity was defined as amount of enzyme that produced decreasing sugars equivalent to 1 µmol of glucose/min.

RESULTS AND DISCUSSION

A lot of effort has been deployed in the quest for cheaper alternative enzyme production techniques in order to produce levan (synthetized by levansucrase enzyme). SSF is one of these methods. There are several factors, which affect SSF processes; most important of which is the nature of solid substrate.

Effect of incubation time on levansucrase production

Fermentation time has a profound effect on enzyme production. Figure 1 shows that the maximum activity (8.25 U/g) obtained after 48 h of SSF. We notice that the enzyme level declined with prolonged incubation, this could be due to loss of moisture or denaturation of the enzyme resulting from pH variation during fermentation. This result is on line with works by Mussatto[15] who obtained the highest level of levansucrase after 48 h. On the other hand, Sangeetha et *al.*[16] found that maximum production of levansucrase by *A. oryzae* in SSF was 8 h.

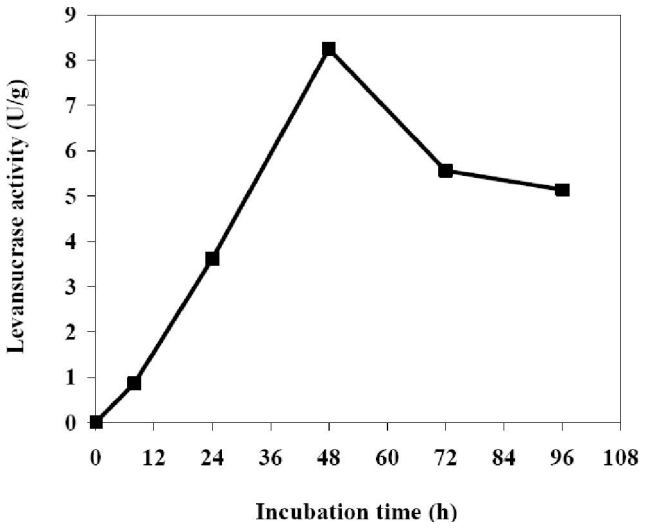

Figure 1 : Effect of incubation period on levansucrase production

Effect of different solid substrate on levansucrase production

The nature of solid substrate is the most important factor in solid state fermentation.

This not only supplies the nutrient to the culture but also serves as an anchorage for the microbial cells[17]. The selection of a substrate for solid state fermentation depends on several factors mainly related to cost, availability, consistency, stability, ease of handling and of course the effect on the production process.

From the various solid substrate used in the present study, date proved to be most suitable for the coloniza-

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tion of *B. licheniformis*, as indicated by the maximum visible growth on the surface of substrate. The highest enzyme activity was obtained in date (15U/g) (Figure 2). This is possibly due to its richness in sugars or probably to the presence of minerals such as calcium which has been reported as an activator of levansucrase activity[18]. However, waste dates are a cheap solid substrate and no research is so far reported to have investigated it. The nature of solid substrate is the most important factor affecting solid state fermentation processes. The selection of a substrate depends on several factors mainly related with cost and availability.

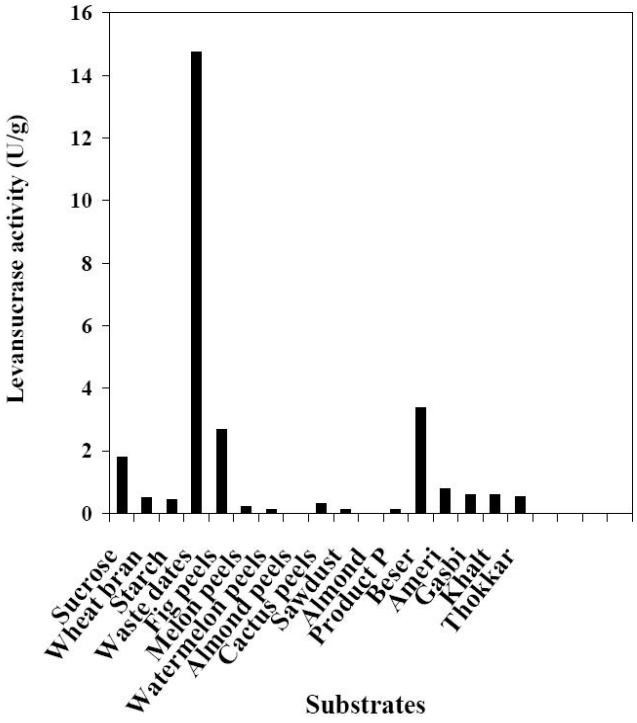

Figure 2 : Effect of different solid substrates on levansucrase production

Several substrates including agricultural crop residues or industrial waste have been used by various works on solid state fermentation. Sangeetha et *al.*[16] studied the production of levansucrase by *A. oryzae* using a great variety of agricultural by-products. They found that the best results were obtained when rice bran, wheat bran, spent coffee and spent tea are used supplemented with yeast extract and complete synthetic media. When the culture was grown on sucrose, fig peels and waste dates, the colonies had a transparent mucoid appearance and made the broth extremely viscous. This proves that levan was produced.

Effect of waste dates on the levansucrase production level

The effect of waste date concentration on enzyme production is shown in Figure 3. We notice that 6 g waste dates in the fermentation medium yielded maximum enzyme activity (16U/g). A further increase in substrate did not increase the enzyme yield significantly.

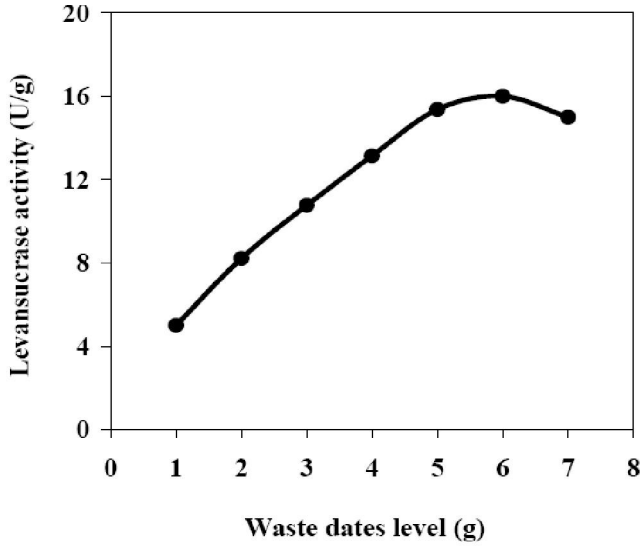

Figure 3 : Effect of waste dates on levansucrase production

Effect of pH on levansucrase extraction from SSF

PH is one of most important factors for any fermentation process. It depends on microorganism because each microorganism possesses a pH range for its growth and activity with an optimum value within this range[19]. The optimal levansucrase production was reached at an initial pH of 6.0 (Figure 4). Increasing or decreasing pH resulted in the decrease in the growth of product fermentation. The pH of the medium to which the enzyme is exposed affects the ionization state of its amino acids, affecting its primary and secondary structure, thus controlling its activity[20]. Yun *et al.*[21] obtained same pH optimum for levansucrase produced by *A. pullulans*.

Effect of temperature on levansucrase extraction from SSF

Maximum yield of enzyme was obtained at 40°C (Figure 5), but at a higher temperature the yield was less. This may be due to the inhibitory effect of temperature on enzyme activity and make it less stable. Song et *al.*[22] reported the same optimum temperature for levansucrase produced by *S. salivarius*.

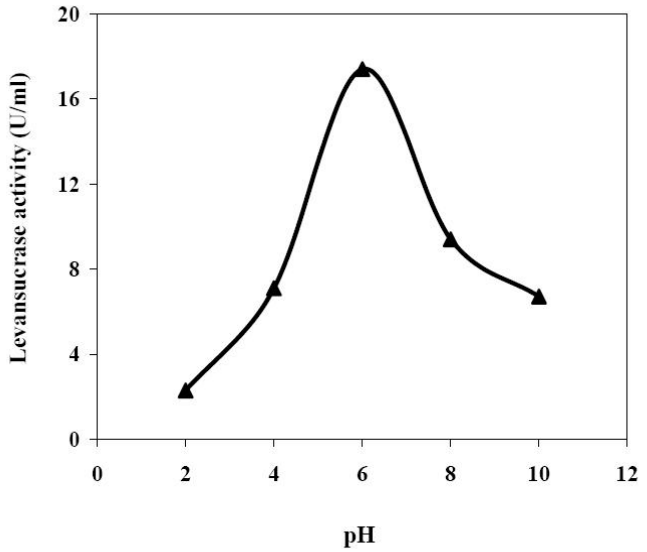

Figure 4 : Effect of pH on levansucrase extraction from solid state fermentation

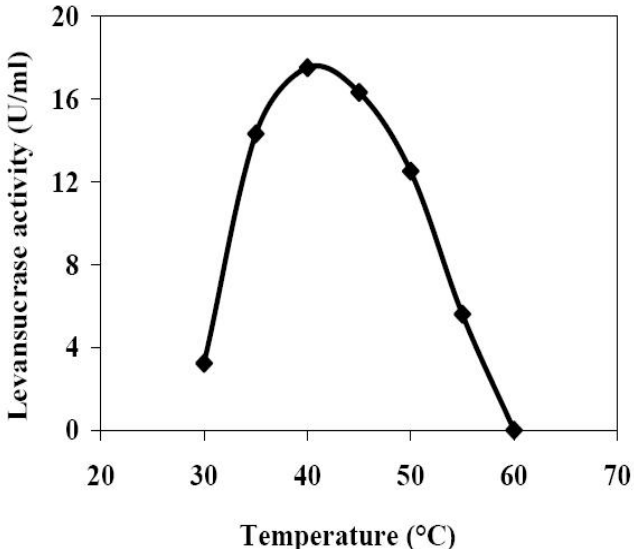

Figure 5 : Effect of temperature on levansucrase extraction from solid state fermentation

Effect of moisture content on levansucrase production

Moisture content is a critical factor for SSF processes because this variable has influence on growth and biosynthesis and secretion of different metabolites. In this study the optimum moisture content for the enzyme production was found to be 80% (Figure 6). Similar results were reported by Ahmed[23]. The critical importance of moisture level in SSF media and its influence on the biosynthesis of enzymes has been attributed to the interference of moisture in the physical properties of solid particles. The optimum moisture content for growth and substrate utilization depends on the organism and the substrate used for cultivation.

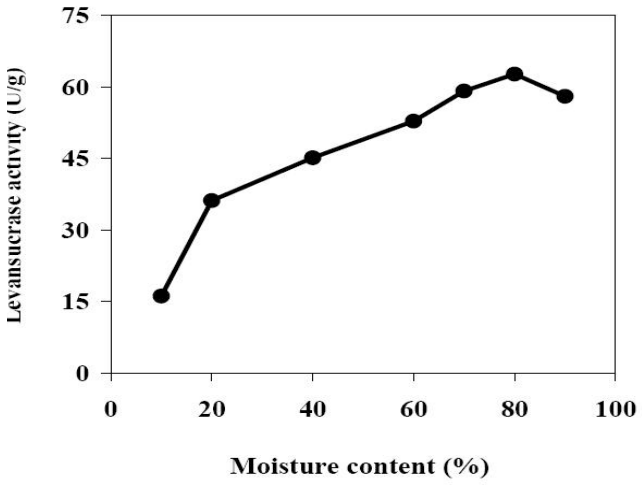

Figure 6 : Effect of moisture content on levansucrase production

Effect of solvents type on levansucrase extraction from SSF

Solid state fermentation is fermentation in the absence of free liquid. Recovery of the fermentation product requires its extraction from the solid fermented medium. The extraction was done by organic and inorganic solvents in addition to distilled water. According to the results (Figure 7), it is clear that among all the solvents, distilled water gave the best extraction of levansucrase from the fermented solids. This might be due to dissolution of the all media broth in distilled water which then becomes salt solution and hence able to extract enzyme protein from fermented biomass. Distilled water is a common extractant (available, safe and low cost) used by other works[24].

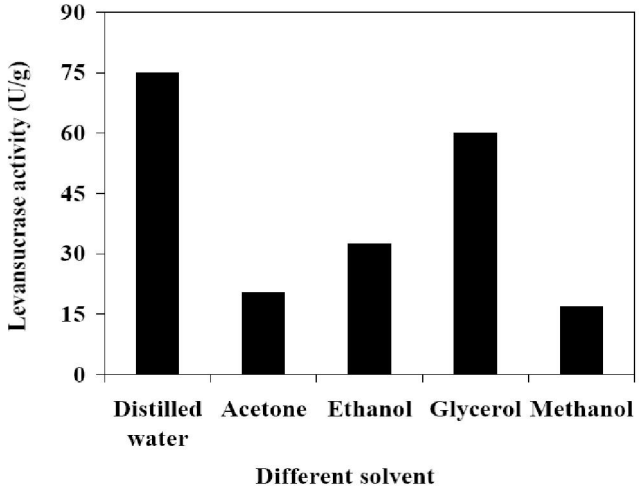

Figure 7 : Effect of solvent type on levansucrase extraction from solid state fermentation

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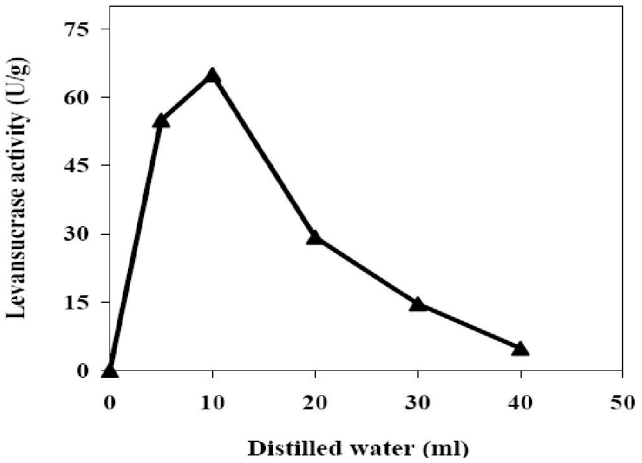

Figure 8 : Effect of distilled water volume on levansucrase extraction from solid state fermentation

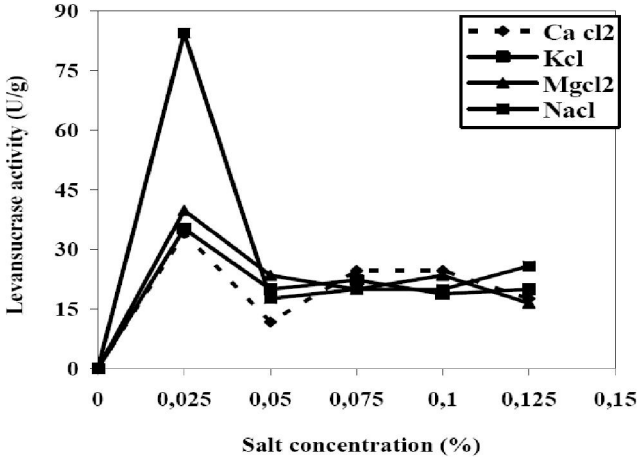

Figure 9 : Effect of inorganic salt solutions on levansucrase extraction from solid state fermentation

Effect of distilled water volume on levansucrase extraction from SSF

In solid state fermentation system free flowing solvent is very much limited. Thus, adequate amount of distilled water is required to leach out the existing enzyme. The results (Figure 8) show that highest levansucrase extraction is reached at ratio 10 ml distilled water. According to Aikat and Bhattacharyya[25], the amount of solute increases in parallel with the increase of solvent volume. The decrease in enzyme extraction when lower volume of solvent was used might be due to insufficient solvent volume to penetrate the solid fermented mass. Excessively large volume of extractant used for greater extraction would also make the obtained enzyme solutions too diluted to be profitably utilized.

Effect of inorganic salt solutions on levansucrase extraction

Among all salt solutions tested (Figure 9), sodium chloride (0.05 %) gave the best extraction of enzyme from fermented solid. Increasing extraction up to 0.05 % probably is due to the salting-in effect of electrostatic effect of salt[26]. This result is on line with the one obtained by Wang[27] who extracted proteolytic enzyme from fermented biomass by elution with sodium chloride.

CONCLUSION

Solid state fermentation is a very promising cultivation technique for the production of industrially-relevant enzymes such as levansucrases, especially by means of agro-wastes as support-substrates like waste dates. The produced enzyme showed maximum activity when date was used as a substrate at 40°C. The production process can be further improved by optimizing the fermentation and culture conditions in order to achieve better yields and reduce the cost. The produced enzyme obtained in this style has some advantages for further studies such as stabilization and immobilization.

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REFERENCES

[1] S.Rhee, K.Song, C.Kim, B.Park, E.Jang, K.Jang; Levan, In A. Steinbüchel 795 (Ed); Biopolymers; polysaccharides, New York: Weinheim, 351-377 **(2002)**.

[2] J.Yun; Enzyme Microb.Technol., **19**, 107-117 **(1996)**.

[3] I.Dahech, K.S.Belghith, H.Belghith, H.Mejdoub; International Journal of Biological Macromolecules, **51**, 329-335 **(2012)**.

[4] K.S.Belghith, I.Dahech, K.Hamden, A.Feki, H.Mejdoub, H.Belghith; International Journal of Biological Macromolecules, **50**, 1070-1074 **(2012)**.

[5] M.Padalino, D.Perez-Conesa, R.López-Nicolás, C.Frontela-Saseta, G.Berruezo; International Dairy Journal, **27**, 27-33 **(2012)**.

[6] A.Pandey, C.R.Soccol, J.A.Rodriguez-Leon, P.Nigam; Fundamentals and Applications; Solid state fermentation in biotechnology, 1st Edition, 8-29 **(2001)**.

[7] S.G.Prapulla, V.Subhaprada, N.G.Karanth; Advances in Appl.Microbiol., **47**, 299-343 **(2000)**.

[8] C.Aranda, A.Robledo, O.Loera, J.C.Contreras-Esquivel, R.Rodriguez, C.N.Aguilar; Food Technol.Biotech., **44**, 229-233 **(2006)**.

[9] A.F.Abdel-Fattah, D.A.R.Mahmoud, M.A.T.Esawy; Current Microbiol, **51**, 402-407 **(2005)**.

[10] S.Palit, R.Banerjee; Brazil Arch.Biol.Technol., **44**, 107-111 **(2001)**.

[11] S.S.Yang, W.F.Chiu; Microb.Int.Cong.Microbiol, **14**, 283-286 **(1987)**.

[12] K.S.Belghith, I.Dahech, H.Belghith, H.Mejdoub; International Journal of Biological Macromolecules, **50**, 451-458 **(2012)**.

[13] P.E.W.Martin, G.R.Lohr, D.H.Deam; J.Bacteriol, **145**, 980-983 **(1981)**.

[14] H.Yanase, H.Fukushi, N.Ueda, Y.Maeda, A.Toyoda, K.Tonomura; Agricultural and Biological Chemistry, **55**,1383-1390 **(1991)**.

[15] S.I.Mussatto, J.A.Teixeira; Biochem.Eng.J., **53**, 154-157 **(2010)**.

[16] P.T.Sangeetha, M.N.Ramesh, S.G.Prapulia; Appl.Microbiol.Biotechnol., **65**, 530-537 **(2004)**.

[17] B.K.Lonsane, N.P.Ghildyal, S.Budiatman, S.V.Ramakrishna; Enz. and Micro.Technol., **7**, 258-265 **(1985)**.

[18] J.Hsiu, E.Fischer, E.A.Stein; Biochemistry, **3**, 61-66 **(1964)**.

[19] H.K.Sodhi, K.Sharma, J.K.Gupta, S.K.Soni; Proc.Biochem., **40**, 525-524 **(2005)**.

[20] S.Kokab, M.Asghar, K.Rehman, M.J.Asad, O.Adedyo; Inter.J.Agric.Biol., **5**, 36-39 **(2003)**.

[21] J.W.Yun, K.H.Jung, J.W.Oh, J.H.Lee; Appl.Biochem.Biotechnol., **24**, 299-308 **(1990)**.

[22] D.D.Song, N.A.Jocoues; Biochem.J., **341**, 285-291 **(1999)**.

[23] S.A.Ahmed; J.Appl.Sci.Res., **10**, 1199-1204 **(2008)**.

[24] S.Malathi, R.Chakraborty; Appl.Environ.Microbiol., **57**, 712-716 **(1991)**.

[25] K.Aikat, B.C.Bhattacharyya; πβοχ Βιοχηεμ, **35**,907-914 **(??2000)**.

[26] H.A.Shata; Pol.J.Microbiol., **54**, 241-247 **(2005)**.

[27] H.L.Wang; J.Bacteriol., **93**, 1794-1799 **(1967)**.