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Comparison of Hydrolysis Abilities onto Soluble and Commercial Raw Starches of Immobilized and Free *B. amyloliquefaciens* α-amylase

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ABSTRACT

Immobilized *Bacillus amyloliquefaciens* α-amylase in calcium alginate beads was used for the effective hydrolysis by using various amounts of soluble potato and various commercial raw starches (potato, maize, wheat, rice), and compared with those of free enzyme. The highest activity was obtained in the presence of commercial raw potato of 4% w/v with immobilized amylase (9740 IU/ml). Immobilized enzyme showed better thermal stability than free enzyme at high temperature. TLC analysis of starches hydrolysis products after 1 h incubation showed the formation of glucose by free amylase and maltose by immobilized amylase

Key Words: *B. amyloliquefaciens*, α-amylase, Calcium Alginate, Starch Hydrolysis, Thin Layer Chromatography

INTRODUCTION

Starch, the main component of many agricultural products, e.g. corn (maize), potatoes, rice and wheat, is deposited in plant cells as reserve material for the organism (Polaina and MacCabe 2007). Starch production by land plants is of the order of $2x10^{10}$ t/year, providing 4/5 of the world's food calculated as calories (Sivak and Preiss 1998). Natural Starches are processed to yield different end products which find many industrial applications. Traditionally, processing is carried out by acid treatment and heating. Enzymatic hydrolysis of starch has now replaced acid hydrolysis in more than 75% of starch hydrolysing processes due to several advantages, not the least of which is its high yield (Tonkova 2006). Starch is hyrdolyzed to glucose, maltose and maltooligosaccarides by α- and β-amylases and other related enzymes. Starch-degrading enzymes are gaining more importance among the industrial enzymes because of the importance of starch, sugar and other products in modern biotechnology era (Prakasham et al. 2007). Amylases are widely used in different process in the food, paper, textile, pharmaceutical industries, distillery and brewing industries (Nigam and Sing 1995). Products obtained during starch hydrolysis by amylases are used in a wide variety of foodstuffs: soft drinks, confectionery, meats, packed products, ice cream, sauces, baby food, canned fruit, preserves, etc. The industrial degradation of starch is usually initiated by α-amylases (EC 3.2.1.1; α-1,4-D-glucan glucanohydrolase) a very common enzyme in microorganisms. Especially, *Bacillus amyloliquefaciens*, *B. stearothermophilus*, *B. subtilis* ve *B. licheniformis* are very important α-amylase sources and becuse their enzymes are thermostable, they are used in gelatinization of starch (Uhling 1998).

The use of enzymes in a soluble or free form must be considered as very wasteful because the enzyme generally cannot be recovered at the end of the reaction. A valuable area of enzyme technology is that concerned with the immobilization of enzymes on insoluble polymers, such as membranes and particles, acting as supports for the enzyme activity (Smith 1998). For industrial applications, the immobilization can offer several advantages, including repeated usage of the enzyme, ease of product separation and improvements in enzyme stability. Enzyme stabilization will thus continue to be a key issue in biotechnology (Ahmed et al. 2008a). Effective enzyme immobilization can be achieved using several techniques including adsorption to insoluble materials, entrapment in polymeric matrix encapsulation, cross linking with a bifunctional reagent, or covalent linking to an insoluble carrier (Kara et al 2005).

Encapsulation technology has been designed to entrap materials within a semi-permeable polymeric membrane and/or a gel matrix (Elcin and Elcin 2000). Basically, there are two main advantages of this immobilization method, i.e., the particle structure allows contact between the substrate and enzyme to be achieved and, additionally, it is possible to immobilize several enzymes at the same time (Hari et al. 1996). Encapsulation in calcium-alginate gels occurs under very mild conditions and is characterized by low cost and ease of use.

In the present study, α-amylase produced from *B. amyloliquefaciens* was immobilized in calcium alginate gel capsules. The hydrolysis ability of immobilized α-amylase on various starchy substrates

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were investigated and compared with free α-amylase. Product profile of starches has been determined by thin layer chromatography. Some properties of immobilized enzyme were examined and compared with those of free enzyme.

MATERIALS AND METHODS

Strain and culture conditions

A new strain of *B. amyloliquefaciens* was obtained from Biochemicals plant (ORBA, Istanbul). Basal medium for liquied culture consists of (%): starch, 1; peptone, 0.5; corn steep liquor, 0.5; $(NH_4)_2SO_4$, 0.8; $MgSO_4.7H_2O$, 0.2; $CaCI_2.2H_2O$, 0.05; K_2HPO_4, 1.4; KH_2PO_4, 0.6, and the pH was adjusted to 7.0 before autoclaving. Cultivation was carried out at 37 ^oC on a rotary shaker operated at 150 rpm for 64 h. Cells were removed by centrifugation at 6000 rpm for 10 min. The supernatant was used as the source of the enzyme.

Enzyme activity

α-Amylase activity was assayed by the starch-iodine method (Yoo et al. 1987). One enzyme unit was defined as the amount of enzyme that hydrolyzed 1 mg of starch (0.1%) in 10 min at 37 °C and pH 5.9 (unit/ml).

Enzyme immobilization

An equal volume of crude enzyme solution and sodium alginate solution were mixed to give a 2% (w/v) final concentration of sodium alginate solution in the mixture. The mixture was taken into a syringe (0.8 mm diameter), and beads were formed by dropping the solution into $CaCl_2$ (5% w/v) solution with gentle stirring at 4°C for 1 h. The formed beads were recovered by filtration and were thoroughly washed with distilled water. The beads were dried using filter paper (Whatman no. 1) followed by exposure to the open air for 1 h before use (Ertan et al. 2007, Rajagopalan and Krishnan 2008). The filtered $CaCl_2$ solution was collected for protein concentration determination. Protein content of free enzyme and the collected washings was determined by using the Lowry method (Lowry et al. 1951). The activity yield was defined as the yield of enzyme that was immobilized in alginate beads (Won et al. 2005).

Enzymatic hydrolysis of starches

Immobilized and free α-amylases were used for the hydrolysis of starches from various sources (soluble potato and commercial raw potato/corn/wheat/rice). Each enzyme was mixed with buffered solution containing 2-10% (w/v) of the starches. The reaction mixture was incubated at 37°C for 10 min. Enzyme activities were assayed by the starch-iodine method. Soluble potato was purchased from Merck. Commercial raw starches were obtained from the local markets.

Effects of some factors on the rate of starch hydrolysis

The effects of some chemical and physical factors such as temperature, pH values, and metal ions on immobilized and free α-amylase activity in the presence of soluble and commercial raw starches which has maximum activity were assayed. Enzyme samples were incubated for 10 min between 45 and 80°C. The optimum pH for the enzymes were determined in a Britton-Robinson buffer system between 4 and 9 pH. Immobilized and free enzymes were incubated with some metal ions of 5 mM. Relative activity was expressed as a percentage of the activity of the untreated control (taken as 100%).

Hydrolysis products of starches

The profile of products formed from various starches hydrolyzed by immobilized and free α-amylase was determined at 1 of reaction time. Products of hydrolysis of starches with α-amylases were visualized on silica gel plates (0.5 mm thick and 20 cm x 20 cm size) by thin layer chromatograhy (TLC).

RESULTS AND DISCUSSION

Hydrolysis of Soluble and Commercial Raw Starch Using Immobilized and Free Enzyme

The α-amylase produced by *B. amyloliquefaciens* strain was immobilized using sodium alginate, and immobilization efficiency observed was 89%. Immobilized α-amylase was used in the hydrolysis of

soluble potato and various commercial raw (CR) starches such as potato, corn, rice and wheat, and compared with free enzyme. Table 1 shows the data to compare the hydrolysis efficiency of free and immobilized enzyme on soluble and CR starches. Immobilized enzyme was more efficient in the hydrolysis of starches than free enzyme. Maximum activities for both of the enzyme forms were determined with soluble potato and CR wheat of 5% (w/v), CR potato and rice of 4% (w/v), and CR corn of 2% (w/v). Soluble and CR potato starches were hydrolyzed much better than the other tested starches by both of amylases. CR corn starch exhibited hydrolysis pattern similar by enzymes, while hydrolysis in the presence of CR wheat increased 1.5 fold by immobilized enzyme. Lower hydrolysis rates were archieved in the presence of CR rice starch. No hydrolysis via free immobilized enzyme in the presence of CR rice (8% and 10%, w/v) was determined. High concentrations of starches were not significant for enzymes. It has been explained that hydrolysis of starches decreases in high starch concentrations because of the restriction on the free movements of both the starch and enzyme molecules (Komolprasert and Ofoli 1991).

Table 1. Enzymatic activities of immobilized and free α-amylase on various starch sources.

Starch Concentration (%)	Sol. Potato		CR. Potato		CR. Corn		CR. Wheat		CR. Rice	
	A*	B*	A*	B*	A*	B*	A*	B*	A*	B*
2	9353	7495	9545	8545	6717	7415	7688	3983	3284	3603
4	9450	7664	9740	8902	6045	6599	8695	5821	4006	3834
5	9643	8422	9447	8456	5843	6451	9153	6128	3004	1917
6	9160	6737	9447	7933	5709	5932	8786	4289	1201	1495
8	7907	5895	9350	6231	5373	5857	8237	4105	0	1303
10	5207	3958	8279	4361	5239	4300	7963	3002	0	690

A*: Immobilized amylase (IU/ml), B*: Free amylase (IU/ml)
Sol.: Soluble, CR: Commercial Raw
Results are the average of a least three complete independent replications

In summary, **s**tarch hydrolysis by immobilized amylase was ranked as CR potato > soluble potato > CR wheat > CR corn > CR rice. For free enzyme, the rank order was CR potato > soluble potato > CR corn > CR wheat > CR rice.

Some workers have been reported that immobilization did lead to more hydrolysis on starches sources. Whereas, some reseaches have been explained that immobilization alters the three-dimensional structure of the enzyme, which causes changes in its affinity toward the substrates, thus increasing its product specificity (Ivanova and Dobreva 1994).

The maximum hydrolysis of potato starch by immobilized α-amylase when compared to rice and corn starch (Konsoula and Kyriakides 2006). Some research indicated that the amount of reducing sugars produced by the immobilized enzyme was highly comparable to the free enzyme. The α-amylase of *B. amyloliquefaciens* have been reported to effectively hydrolyse potato, corn, wheat and rice starch. However, the free enzyme exhibited 85% hydrolysis of raw potato starch (Gangadharan et al. 2009). The highest enzyme activity by immobilized enzyme was determined in soluble potato starch hydrolysis (Ertan et al. 2007). On the other hand, It has been reported that the degree of hydrolysis was about 10–20% less with immobilized α-amylase in one batch reaction. The degree of hydrolysis with immobilized enzyme was lower than that with free α-amylase. This could be due to restricted accessibility of entrapped α-amylase to the substrate (Rajagopalan and Krishnan 2008).

In general, it has been reported that potato starches are capable of digesting by α-amylase. Adsorbtion mechanism of enzyme on starch granules is not still clear, whereas C binding domain was so effective on raw starch sources (Mikami et al. 1999). On the other hand, in our previous study, we have been reported that the number and size of granules, and the starch structure were significant for enzyme attack (Demirkan Sarikaya et al. 2005).

Pysicochemical Properties of Immobilized and Free α-amylase

Some factors such as temperature, pH and metal ions are very effective on starch degradation. In this study, enzyme activity assay was tested at 40, 45, 50, 55, 60, 70 and 80°C to determine the effect of

temperature on hydrolysis of starch sources by immobilized and free ezymes. As shown in Fig. 1, the optimum temperature of immobilized enzyme in the presence of CR potato (4% w/v), wheat (5% w/v) and rice (4% w/v) were 50°C, was 55°C at CR corn (2% w/v), and was 60°C at soluble potato (5% w/v) starch. The optima of free enzyme were found to be 50°C in the presence of CR potato and CR rice, to be 55°C at soluble potato, CR corn and CR wheat starches (Fig. 2). It was established that free enzyme lost its activity at high temperatures. However, immobilized enzyme showed more activity than free one (at 70 and 80°C).

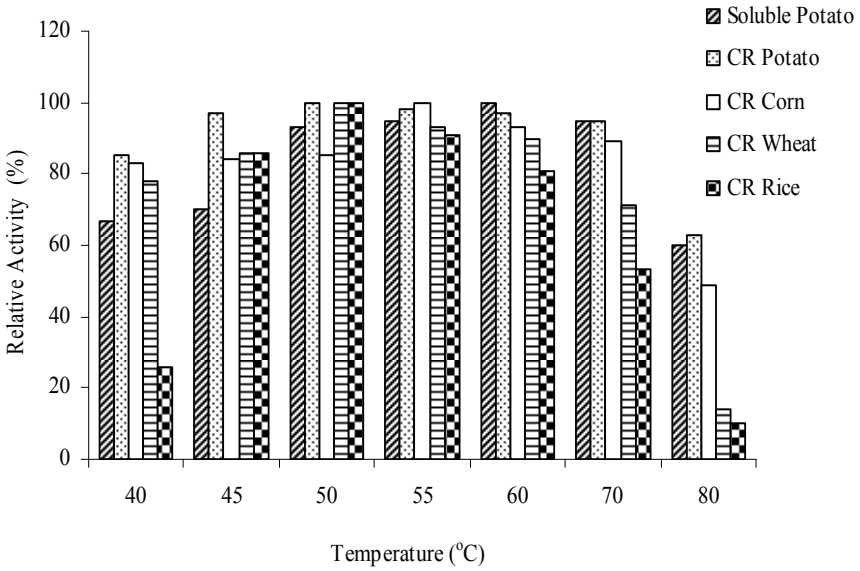

Figure 1. Effect of temperature on immobilized enzyme

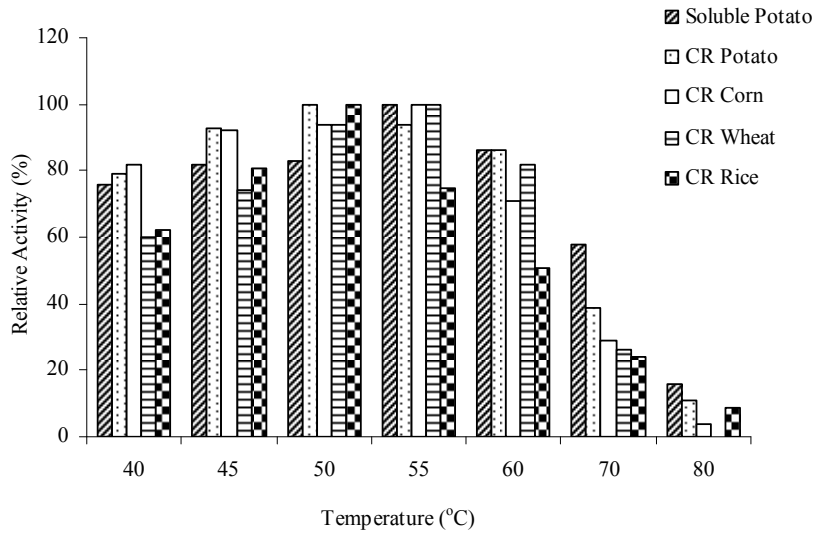

Figure 2. Effect of temperature on free enzyme

The pH is also one of the factors that affect the rate of hydrolysis reaction. Figure 3 shows that the optimum pH values for immobilized amylase in the presence of CR potato and CR corn, CR rice, and soluble potato and CR wheat were 6.0, 6.4, and 6.8, respectively. Whereas, free enzyme had diversity. Accordingly, the pH optima was found to be 6.0 at CR corn and CR rice, to be 6.4 at soluble potato and

CR wheat, to be 6.8 at CR potato (Fig. 4). Immobilized enzyme had more activity at alkali pH than free enzyme. Both enzymes lost up to %90 of its activity in the presence of CR rice starch at pH 8.0.

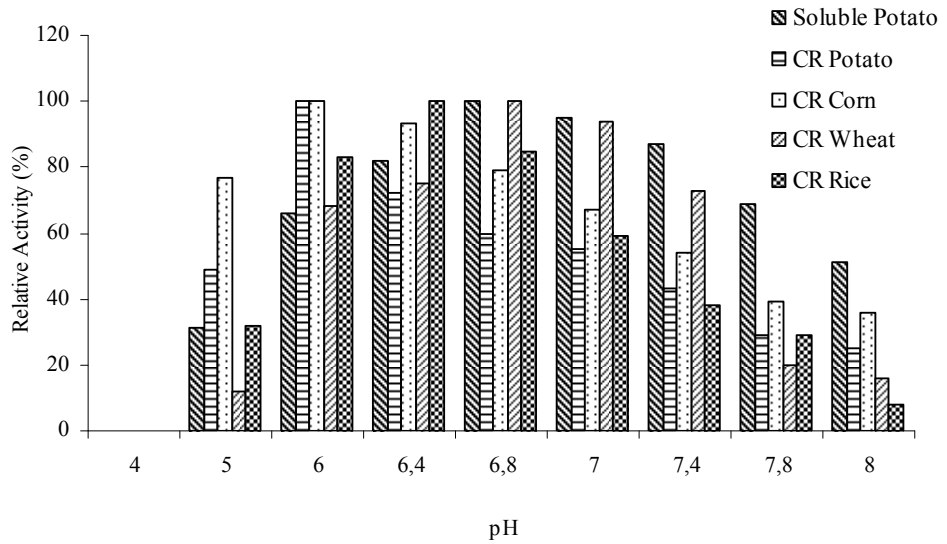

Figure 3. Effect of pH on the activity of immobilized enzyme

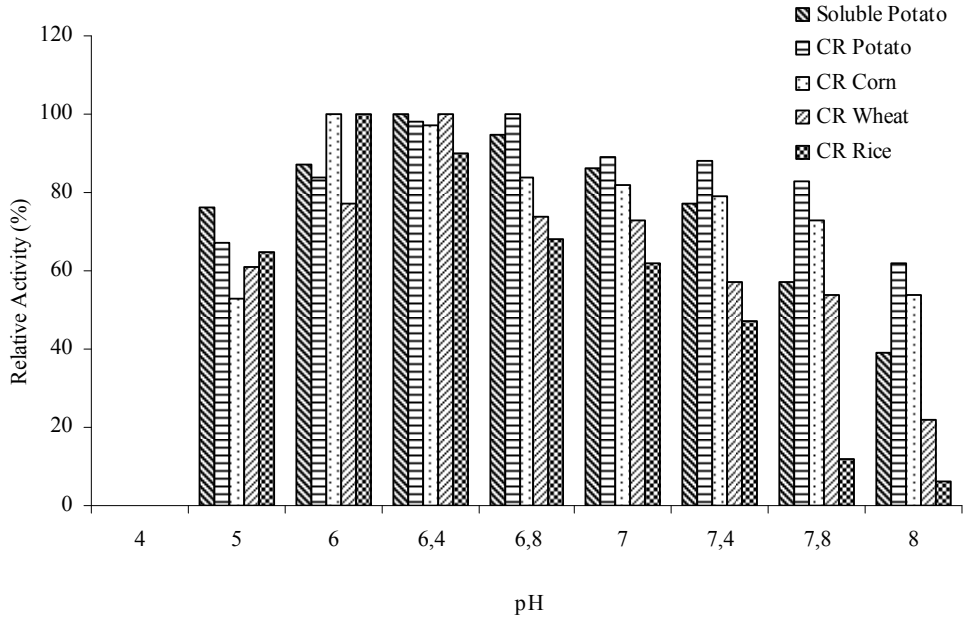

Figure 4. Effect of pH on the activity of free enzyme

Similar findings have also been recorded by other reseachers. Immobilized glycoamylase and pullulanase enzymes in calcium alginate beads and indicated that immobilized form is more stable than free form at 45°C (Roy and Gupta 2004). The free *Halobacterium halobium* α-amylase enzyme was inactive at 60°C, but immobilized form did not lose its activity until 65°C (Patel et al. 1996). Immobilized α-amylase from *Aspergillus oryzae* in calcium alginate beads indicated that immobilized form was more stable compared to free from. While optimum temperature was 54°C for free enzyme, it increased to 60°C for immobilized enzyme (Kumar et al. 2006). Catalytic activities of free and

immobilized enzymes were compared in several temperatures and immobilized enzyme was more stable than free enzyme and had a higher activity (Kahraman et al. 2007).

α-amylase enzyme of *Bacillus* strains was generally tolerated to alkaline pH, and more stabile. It has been reported that free enzyme was inhibited at acidic pH, immobilized form remained more stabile and saved 75-80% of its activity at alkaline pH (Patel et al. 1996). α-amylase from *Aspergillus oryzae* was immobilized in calsium alginate beads and free enzyme was active in optimum pH 5.5-6.0, immobilized enzyme was active in pH 6.0-6.5 (Kumar et al. 2006). It has been reported that immobilized enzymes are significantly more resistant than free form to the conditions such as pH and temperature (Chang et al. 1996, Gangadharan et al. 2009). In this study, we showed that the immobilization matrix might be able to protect the enzyme against denaturation at high temperature and pH.

Amylases are metaloproteins. The activity of amylases was accelerated by some ions. Therefore, the effect of the metal salts on the activity of free and immobilized amylase was investigated. The metal ions (1 mM) indicated different stimulator effects on starches with both of enzyme (Table 2). The activation by metal ions (Mg^{2+}, Mn^{2+}, $Li^{2+}$and Ba^{2+}) became more pronounced with the free enzyme. Metal ions did not increase immobilized enzyme activity. This result may depend on limitation of diffusion of metal ions to the active core of enzyme by capsules. The metal Cu^{2+} caused inhibition on the activity of free enzyme, but the immobilized enzyme showed a insignificant activity in the presence of soluble potato, com.corn, com.wheat, and com.rice. Most α-amylases are inhibited by the metal ions Hg^{2+}, Fe^{3+}, Cu^{2+}, and Zn^{2+} (Ramesh and Lonsane 1990). However, It has been recorded that immobilization protected the enzyme from the inhibition by some metal ions such as Co^{2+}, Cu^{2+} and Fe^{3+} (Ahmed et al. 2008b). Contrary to our observation, It has been reported that the effects of metal ions on enzyme activity and has been found that Na^{+2} and Ca^{+2} had a very activating effect for free and immobilized enzymes. Co^{+2} and Ni^{+2} ions had an inhibiting effect on activity (El-Banna et al. 2007).

Table 2. Effect of different metal ions on residual activity (%) of immobilized and free *B.amyloliquefaciens* amylase activity in the presence of various starches.

Metal Ions (5 mM)	Sol. Potato (5% w/v)		CR. Potato (4% w/v)		CR. Corn (2% w/v)		CR. Wheat (5% w/v)		CR. Rice (4% w/v)	
	A*	B*	A*	B*	A*	B*	A*	B*	A*	B*
None	100	100	100	100	100	100	100	100	100	100
Ca^{+2}	74	108	67	93	85	93	67	81	59	116
Mg^{+2}	96	85	80	109	101	95	100	76	107	124
Mn^{+2}	89	85	76	74	100	100	86	121	70	108
Ba^{+2}	83	111	60	53	65	86	46	88	66	68
Cu^{+2}	11	0	0	0	12	0	9	0	22	0
Li^{+2}	71	131	53	107	80	104	42	103	70	56

A*: Immobilized amylase, B*: Free amylase
Sol.: Soluble, CR: Commercial Raw
Results are the average of a least three complete independent replications

Product Analysis by Thin Layer Chromatography (TLC)

To determine hydrolysis products from various starches by immobilized and free *α*- amylase after 1 of reaction, product distibutions were analysed by thin layer chromatography (TLC). The product distributions of soluble potato, com. potato, com. corn, com. wheat and com. rice by both the enzymes showed differences. With free amylase, the end product profiles of starch hydrolysis were determined as the formation of glucose, maltose, and maltotriose (G1-G3) after 1 h (Fig. 5). The formation of glucose by immobilized amylase did not show after 1h and the major products formed were in the order G2-G6 (Fig. 6). The main hydrolysis product of starches by both amylases were maltose. Immobilized enzyme showed much better hydrolysis than free enzyme and the composition of starch hydrolysate produced by immobilized amylase is different from that free amylase. The level products formed starches by free enzyme and immobilized enzyme were observed as G2>G3>G1 and G2>G3>G5>G6>G4, respectively, after 1 h.

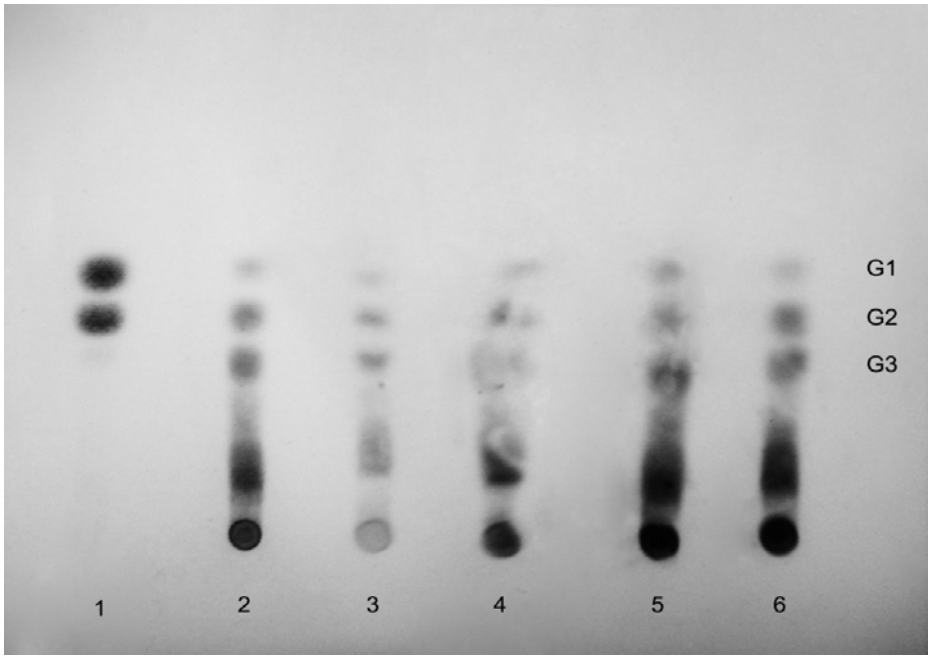

Figure 5. Thin layer chromatographic analysis of the main products from the hdyrolysis of starches by free enzyme at 1 h. 1-Standarts (Glucose and Maltose), 2-Soluble Potato, 3-CR. Potato, 4-CR. Corn, 5-CR. Wheat, 6-CR. Rice
Glucose (G1), Maltose (G2) and Maltotriose (G3)

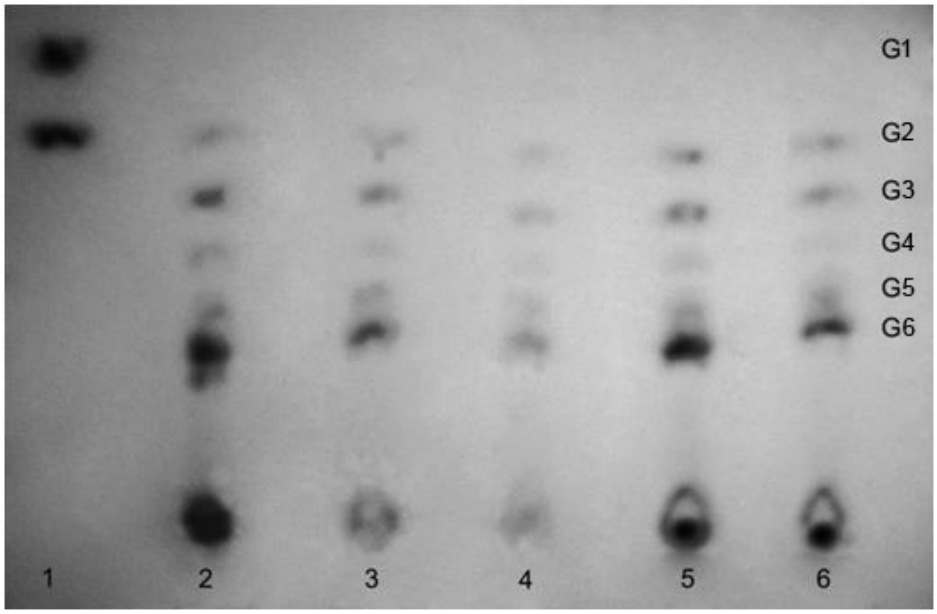

Figure 6. Thin layer chromatographic analysis of the main products from the hdyrolysis of starches by immobilized enzyme at 1 h. 1-Standarts (Glucose and Maltose), 2-Soluble Potato, 3-CR. Potato, 4-CR. Corn, 5-CR. Wheat, 6-CR. Rice.
Glucose (G1), Maltose (G2), Maltotriose (G3), Maltotetrose (G4), Maltopentose (G5) and Maltohexose (G6)

Although there are many reports on the use of immobilized amylase for starch hydrolysis, there are not many studies about the product distribution of soluble and commercial starches.

Using thin-layer chromatographic analysis, the end products of starch hydrolysis detected were glucose and maltose which suggested an endo-mode of action for the amylase (Ray et al. 2008). It has

been reported that the product distribution for starches from rice, wheat and corn by immobilized and free amylase after 7 h was different from soluble and potato starch. Soluble starch and potato starch formed a wide range of maltooligosaccharides (G1–G5). Starches from wheat, rice and corn formed a narrow range of smaller oligosaccharides (G1–G3) as the major products. The major products formed from soluble starch by free *α*-amylase were G3>G2>G4>G1, for immobilized amylase was G2>G3>G4>G5 (Rajagopalan and Krishnan 2008). This observation is contrary to our results, because in this study, all starches showed the same end products. Both enzymes showed different products of starch hydrolysis.

On the other hand, immobilized α-amylase from *Bacillus circulans* GRS 313 in calcium alginate beads has been showed glucose and it has also been explained that glucose was successful for glucose syrup production (Dey et al. 2003). Immobilized α-amylase from *B. licheniformis* hydrolyzed soluble starch to G1, G2, G3, G4 and G5. Saccharid composition of the immobilized α-amylase was different from free enzyme's. While maltotriose, maltopentose and maltohexose occured after the hydrolysis of starch by free enzyme, maltotriose and maltopentose that occured by immobilized enzyme (Shewale and Pandit 2007).

CONCLUSIONS

Immobilization procedure can be carried out in a single-step process under very mild conditions and is therefore compatible with most enzymes. It makes enzymes easy to handle, easy to recover and often enhances stability. The immobilized enzyme had a good advantage to be used in starch degradation industry. α-amylase enzyme from *B. amyloliquefaciens* was successfully immobilized in calcium alginate beads. Hdyrolysis abilities on soluble and commercial starches of different concentrations were investigated and compared with free enzyme. Immobilized enzyme showed high activitiy in the presence of soluble potato, com. potato, com. wheat and com. corn than free enzyme. However, high and low concentrations of starches were not significant for enzymes. The temperature and pH optima of enzymes in the presence of starches obtained maximum activity were different. However, immobilized enzyme showed more activity than free one (at 70 and 80 °C) and had more activity at alkali pH. Metal ions did not increase immobilized enzyme activity. The products formed were the same with starches from various sources and immobilized enzyme. A widely product distibutions were obtained with immobilized enzyme. These results showed that immobilization of α-amylase is very useful for starch hdyolysis and economically produce malto-oligosaccharides.

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REFERENCES

Ahmed SA, Al-Domany RA, El-Shayeb NMA, Radwan HH, and Saleh HA (2008a).
Optimization, Immobilization of Extracellular Alkaline Protease and Characterization of its Enzymatic Properties. Res. J. Agr. Biol. Sci., 4: 434- 446.

Ahmed SA, El-Sayed MEM, Hassan O, Saleh NA, and Goda HA (2008b). Studies on the Activity and Stability of Immobilized *Bacillus acidocaldarius* alpha-amylase. Aust. J. Basic. Appl. Sci., 2: 466-474.

Chang HN, Seong GH, Yoo IK, Park JK, and Seo JH (1996). Microencapsulation of recombinant *Saccharomyces cerevisiae* cells with invertase activity in liquidcore alginate capsules. Biotechnol. Bioeng., 51: 157-162.

Demirkan Sarikaya E, Mikami B, Adachi M, Higasa T, and Utsumi S (2005). α-amylase from *B. amyloliquefaciens*: purification, characterization, raw starch degradation and expression in *E. coli*. Process Biochem., 40: 2629-2636.

Dey G, Singh B, and Banerjee R (2003). Immobilization of α-Amylase Produced by *Bacillus circulans* GRS 313. Braz. Arch. Biol. Techn., 46: 167-176.

El-Banna ET, Ahmed AA, Mohamed IA, and Ibrahim II (2007). Production and Immobilization of α-amylase from *Bacillus subtilis*. Pak. J. Biol. Sci., 10: 2039-2047.

Elcin AE, and Elcin YM (2000). Polycation-coated polyanion microspheres of urease for urea hydrolysis. Artif Cells Blood Substit Immobil. Biotechnol., 28: 95-111.

Ertan F, Yagar H, and Balkan B (2007). Optimization of alpha-amylase immobilization in calcium alginate beads. Prep. Biochem. Biotechnol., 37: 195-204.

Gangadharan D, Nampoothiri KM, Sivaramakrishnan S, and Pandey A (2009). Immobilized bacterial α-amylase for effective hydrolysis of raw and soluble starch. Food Res. Int., 42: 436- 44.2

Hari PR, Chandy T, and Sharma CP (1996). Chitosan/calcium alginate microcapsules for intestinal delivery of nitrofurantion. J. Microencapsul., 13: 319-329.

Ivanova V, and Dobreva E (1994). Catalytic properties of immobilized purified thermostable α-amylase from *Bacillus licheniformis* 44MB82-A. Process Biochem., 29: 607-612.

Kahraman MV, Bayramoglu G, Kayaman-Apohan N, and Gungor A (2007). UV curable methacrylated / fumaric acid modified epoxy as a potential support for enzyme immobilization. React. Funct. Polym., 67: 97-103.

Kara A, Osman B, Yavuz H, Besirilana N, and Denizli A (2005). Immobilization of α amylase on Cu^{+2} chelated poly (ethylene glycol dimethacrylate–vinylimidazole) matrix via adsorption. React. Funct. Polym., 62: 61-68.

Komolprasert V, and Ofoli RY (1991). Starch hydrolysis kinetics of *Bacillus licheniformis* α-amylase. J. Chem. Technol. Biotechnol., 51: 209-223.

Konsoula Z, and Kyriakides ML (2006). Starch hydrolysis by the action of an entrapped in alginate capsules α-amylase from *Bacillus subtilis*. Process Biochem., 41: 343-349.

Kumar RSS, Vishwanath KS, Singh SA, Rao AGA (2006). Entrapment of α-amylase in alginate beads: Single step protocol for purification and thermal stabilization. Process Biochem., 41: 2282-2288.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

Mikami B, Adachi M, Kage T, Sarikaya E, Nanmori T, Shinke R, and Utsumi S (1999). Structure of raw-starch digesting *Bacillus cereus* β-amylase complexed with maltose. Biochemistry., 38: 7050-7061.

Nigam P, and Sing D (1995). Enzymes and microbial systems involved in starch processing. Enzyme Microbial. Technol., 17: 770-778.

Patel S, Bagai R, and Madamwar D (1996). Stabilization of a Halophilic α-Amylase by Calcium Alginate Immobilization. Biocatal. Biotransform., 14: 147-155.

Prakasham RS, Rao CS, Rao RS, and Sarma PN (2007). Enhancement of acidamylase production by an isolated *Aspergillus awamori*. J. Appl. Microbiol., 102: 204-211.

Polaina J, and MacCabe AP (2007). Industrial Enzymes: Structure, function and Applications. Dordrecht, Springer, Berlin.

Rajagopalan G, and Krishnan C (2008). Immobilization of malto-oligosaccharide forming α-amylase from *Bacillus subtilis* KCC103: properties and application in starch hydrolysis. J. Chem. Technol. Biotechnol., 83: 1511-151.7

Ramesh MV, and Lonsane BK (1990). Effect of metal salts and protein modifying agents on activity of thermostable α-amylase produced by *Bacillus lichiniformis* M27 under solid state fermentation. Chem. Mikrobiol. Technol. Lebensm., 12: 129-136.

Ray RC, Kar S, Nayak S, and Swain MR (2008). Extracellular α-amylase Production by *Bacillus brevis* MTCC 7521. Food Biotechnol., 22: 234-246.

Roy I, and Gupta MN (2004). Hydrolysis of starch by a mixture of glucoamylase and pullulanase entrapped individually in calcium alginate beads. Enzyme Microbial. Technol., 34: 26-32.

Shewale SD, and Pandit AB (2007). Hydrolysis of soluble starch using *Bacillus licheniformis* α-amylase immobilized on superporous CELBEADS. Carbohyd. Res., 342: 997-1008.

Sivak MN, and Preiss J (1998). Starch: Basic Science to Biotechnology. Advanced in Food and Nutrition Reserach. Academic, New York.

Smith EJ (1998). Biotechnology: Studies in Biology. Third edition. University press, Cambridge.

Tonkova A (2006). Microbial starch converting enzymes of the α-amylase family. In: Microbial Biotechnology in Horticulture (Eds.: R.C. Ray and O.P. Wards). Science Publishers, Enfield, pp. 421-472.

Uhling H (1998). Industrial enzymes and Their applications. John Wiley & Sons, New York.

Won K, Kim S, Kim KJ, Park HW, and Moon SJ (2005). Optimization of lipase entrapment in Ca-alginate gel beads. Process Biochem., 40: 2149-2154.

Yoo YJ, Hong J, and Hatch R (1987). Comparison of α-amylase activities from different assay methods. Biotechnol. Bioeng., 30: 147-151.