Food Research International 42 (2009) 436–442

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Immobilized bacterial α-amylase for effective hydrolysis of raw and soluble starch

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ARTICLE INFO

Article history:
Received 14 November 2008
Accepted 3 February 2009

Keywords:
Immobilization
Bacillus amyloliquefaciens
α-Amylase
Calcium alginate
Raw starch
Starch hydrolysis

ABSTRACT

The major concern in an enzymatic process is the instability of the enzyme under repetitive or prolonged use and inhibition by high substrate and product concentration. Immobilization is a very effective alternative in overcoming problems of instability and repetitive use of enzymes. Entrapment method of immobilization is advantageous over other methods as they do not involve chemical modification of the enzyme. α-Amylase produced by *Bacillus amyloliquefaciens* ATCC 23842 was immobilized in calcium alginate beads and used for the effective hydrolysis of soluble and raw potato starch which was comparable to the free enzyme. The levels of parameters (sodium alginate, calcium chloride and curing time) that significantly influence the immobilization of α-amylase in calcium alginate were analyzed and optimized using response surface methodology. Reactor studies were performed to study the reusability and operational stability of the beads. The alginate beads retained more than 60% of their initial efficiency after five batches of successive use and 40% of efficiency was exhibited in the 6th and 7th batch run of 6 h duration.

1. Introduction

Amylase has a great deal of application in starch saccharification. The amylolytic enzymes find a wide spectrum of applications in food industry for production of glucose syrups, high fructose corn syrups, maltose syrups, reduction of viscosity of sugar syrups, reduction of haze formation in juices, solubilization and saccharification of starch for alcohol fermentation in brewing industries, and also find a wide range of application in baking, paper, textile and detergent industry (Sivaramakrishnan, Gangadharan, Nampoothiri, & Pandey, 2006). In most cases the enzymatic process is inhibited by high substrate and product concentration and also instability of the enzyme under repetitive or prolonged use. Immobilization is an important technique for continuous and repeated use of enzymes in industrial application and also rapid separation of the enzyme from the reaction medium. The above features would be important in the development of an economically feasible bioreactor for the starch hydrolysis industry thus immobilizing α-amylase would be of great importance. The general methods employed for immobilization are entrapment, microencapsulation, copolymerization, cross linking, physical adsorption, chemical attachment and covalent binding (Hasirci, Aksoy, & Tumturk, 2006; Markweghanke, Lang, & Wagner, 1995; Mozhaev et al., 1989; Rajagopalan & Krishnan, 2008; Reshmi, Sanjay, & Sugunan, 2006).

Immobilization by physical adsorption on inorganic materials such as porous silica (Cao, Bornscheuer, & Schmid, 1999) clay (Sanjay & Sugunan, 2005a, 2005b) and collagen (Groom, Meising, & White, 1988) has been reported. The immobilization of α-amylase adsorption on alumina has been reported for the hydrolysis of starch to low molecular weight carbohydrates (Reshmi et al., 2006). Functional glass beads were used as support for covalent attachment. Poly(dimer acid-co-alkyl polyamine) particles activated by using various chemicals such as carbodiimide, ethylene diamine, and hexamethylene diamine have been studied as support materials for covalent immobilization of α-amylase (Hasirci et al., 2006). Covalent immobilization of amylase has been carried out using UV-curable methacrylated/fumaric acid modified cycloaliphatic epoxide as a rigid support material. Even though immobilization of enzymes via covalent binding on solid supports have some advantages such as increased thermal and storage stability problems of diffusional resistance were overcome in the case of chemical crosslinked matrix by the use of super porous CELBEADS (Satish, Shewale, & Pandit, 2007). Immobilization techniques that involve chemical modification may cause detrimental effects or may be stressful to the enzyme which is overcome by the entrapment method. Among different immobilization techniques, entrapment in calcium alginate gel offers many advantages due to its simplicity and non-toxic character (Gombotz & Wee, 1998). The gelation characteristics can be altered easily thus capsule characteristics such as the thickness or permeability to different substrates of the gel membrane can be easily controlled (Bladino, Macias, & Cantero, 2002). Alginate beads have been successfully used for the entrapment of α-amylase of *Bacillus subtillus* and effectively used for starch hydrolysis (Rajagopalan & Krishnan, 2008). The present study has exploited the simple technique of

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doi:10.1016/j.foodres.2009.02.008

entrapment using calcium alginate for the immobilization of α-amylase produced by *Bacillus amyloliquefaciens* ATCC 23842 and also performs reactor studies to confirm their operational stability and reusability.

2. Materials and methods

2.1. Microorganism and enzyme production

B. amyloliquefaciens ATCC 23842 was used for the present study. The strain was grown on nutrient agar (Hi-media, Mumbai, India) slants at 37 °C for 24 h and sub-cultured every 2 weeks. The production medium was composed of 12.5% w/v of wheat bran and groundnut oil cake (1:1) supplemented with $MgSO_4$ 0.05 M, NH_4NO_3 0.2 M, KH_2PO_4 0.05 M, $CaCl_2$ 0.0275 M. The production was carried out in 250 ml Erlenmeyer flask inoculated with 10^6 CFU/ml of 18 h old culture and incubated at 37 °C with 180 rpm. The sample was withdrawn after 42 h fermentation, centrifuged at 2862*g* for 20 min and the clear supernatant collected was used as crude enzyme.

2.2. Immobilization of α-amylase

The entrapment of the enzyme in calcium alginate beads were carried out by thoroughly mixing the enzyme with sodium alginate (1:1 v/v) by mild shaking on a rotary shaker. The alginate–enzyme mixture was taken in a syringe (0.7 × 32 mm) fitted with a needle and the solution was added drop by drop from the syringe into the $CaCl_2$ solution. The beads were cured at 4 °C and were filtered and washed with distilled water thoroughly to remove any unbound protein. The unbound enzyme activity or enzyme leakage was determined in the curing and wash out solution.

The levels of parameters (sodium alginate, calcium chloride and curing time) that influence the immobilization of α-amylase in calcium alginate beads significantly were analyzed and optimized using response surface methodology. The Box–Behnken design (Box & Behnken, 1960) was used in the present study and the experimental plan (Table 1) consisted of 17 trials. The independent variables were studied at three different levels, low (−1), medium (0) and high (+1). All the experiments were done in triplicate and the reducing sugar produced and binding efficiency of the enzyme was determined and taken as the dependent variables or response (Y_1 and Y_2). The binding efficiency of the enzyme (Y_2) was calculated using the following equation

$$\text{Enzyme binding} = (E_A - E_B/E_A) \times 100,$$

Table 1
Box–Benken design for the optimization of variables for effective immobilization of α-amylase of *Bacillus amyloliquefaciens*.

Run	A: Sodium alginate (%)	B: Calcium chloride (M)	C: Curing time (h)
1	3	0.1	6
2	3	0.1	3
3	1	1	4.5
4	5	0.55	3
5	3	1	3
6	3	0.55	4.5
7	3	0.55	4.5
8	3	0.55	4.5
9	3	0.55	4.5
10	5	1	4.5
11	3	1	6
12	3	0.55	4.5
13	5	0.1	4.5
14	5	0.55	6
15	1	0.55	6
16	1	0.1	4.5
17	1	0.55	3

Table 2
Responses obtained for Box–Behnken design.

Run	Efficiency of bead formation Y_1 (reducing sugar mg/ml)	Binding efficiency Y_2 %
1	179	60
2	199	62
3	211	74
4	275	63
5	270	88
6	321	80
7	319	79
8	325	83
9	314	81
10	241	68
11	234	55
12	328	68
13	220	45
14	204	65
15	196	38
16	168	35
17	192	66

where E_A is enzyme added (U/ml) and E_B is the unbound enzyme (U/ml).

The second order polynomial coefficients were calculated and analyzed using the 'Design Expert' software (Version 6.0, Stat-Ease Inc., Minneapolis, USA) statistical package. The general form of the second degree polynomial equation (1) is:

$$Y_i = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

where Y_i is the predicted response, X_iXj are input variables which influence the response variable Y; β_o is the offset term; β_i is the ith linear coefficient; β_{ii} the ith quadratic coefficient and β_{ij} is the ijth interaction coefficient.

Table 3
Analysis of variance (ANOVA) for bead formation efficiency.

Source	Sum of squares	DF	Mean square	*F*-value	Prob > *F*
Model	51357.52	9	5706.391	64.122	<0.0001
A	3741.125	1	3741.125	42.038	0.0003
B	4512.5	1	4512.5	50.706	0.0002
C	1891.125	1	1891.125	21.250	0.0025
A2	13957.39	1	13957.39	156.837	<0.0001
B2	12198.44	1	12198.44	137.072	<0.0001
C2	9330.761	1	9330.761	104.848	<0.0001
AB	121	1	121	1.360	0.2818
AC	1406.25	1	1406.25	15.802	0.0054
BC	64	1	64	0.719	0.4245
Residual	622.95	7	88.99286		
Lack of fit	505.75	3	168.5833	5.754	0.0620

CV – 3.82; R^2 – 0.99.

Table 4
Analysis of variance (ANOVA) for binding efficiency.

Source	Sum of squares	DF	Mean square	*F*-value	Prob > *F*
Model	3386.479	9	376.2755	6.439	0.0113
A	98	1	98	1.677	0.2364
B	861.125	1	861.125	14.736	0.0064
C	465.125	1	465.125	7.960	0.0257
A2	1008.318	1	1008.318	17.255	0.0043
B2	219.7921	1	219.7921	3.761	0.0936
C2	94.00263	1	94.00263	1.609	0.2452
AB	64	1	64	1.095	0.3301
AC	225	1	225	3.850	0.0905
BC	240.25	1	240.25	4.111	0.0822
Residual	409.05	7	58.43571		
Lack of fit	270.25	3	90.08333	2.596061	0.1897

CV – 11.71; R^2 – 0.892.