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Optimization of Acidic Isoamylase Production by Isolated *Bacillus coagulans*

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Abstract: Background: *Bacillus coagulans* isolated from soil produced extracellular isoamylase. **The Purpose and Context:** The present study aimed to optimize isoamylase production under acidic conditions from *Bacillus coagulans* strain isolated from soil sample. The optimized conditions for isoamylase production was found when liquid shaking culture was incubated 120 h at 30°C with initial pH 5. **Results:** The optimum inoculum size on the production of isoamylase was 100 50 mL^{-1} medium. Soluble starch at 0.75% was the best inducer for enzyme production as a carbon source and glycine at 1.078% was better than the other nitrogen sources for production of enzyme.

Key words: Optimization, acidic, isoamylase production, enzyne production, *Bacillus coagulans*

INTRODUCTION

The use of enzymes is preferred as it offers a number of advantages including improved yields and favourable economics (Gurudeeban *et al.,* 2011; Satyanarayana *et al*., 2004). Isoamylase was first discovered in autolysed brewers' yeast. This intracellular enzyme has also been found in baker's yeast (Burhan *et al*., 2003). An extracellular yeast isoamylase has been reported by Gazi *et al*. (2004) from *Lypomyces kononenkoae* by identified an extracellular isoamylase produced by *Escherichia coli* then from *Pseudomonas amyloderamosa* which was then purified and characterized (Regulapati *et al*., 2007; Ray, 2004). Isoamylase (amylopectin-6-glucanohydrolase or glycogen-6-glucanohydrolase, EC 3.2.1.68) catalyzes the hydrolysis of α-1, 6-glucosidic linkages of amylopectin and related polysaccharides (Kubo *et al*., 2010; Shaptadvipa and Sarma, 2009). Isoamylase is useful not only for the structural analysis of polysaccharides and derived oligosaccharides (Herrero-Martinez *et al*., 2004) but also for the starch industry (David Stevenson *et al.,* 2007) in producing glucose, maltose and higher oligosaccharides from starch with the action of exo-type hydrolases. Isoamylase also can be used in conjunction with CGTase to enhance the production of cyclodextrins from starch (Yap *et al*., 2010; Zofia, 2007) and to improve their solubility and hemolytic product through the reversed action of enzyme. The genus *Bacillus* has been in use in the biotechnology industry for a very long time with a number of new cultures exhibiting a variety of benefits to humans (Sudha *et al*., 2010; Walton *et al.,* 2010). The objective of this study is to produce isoamylase from *Bacillus coagulans,* furthermore optimize the nutritional factors of its production.

MATERIALS AND METHODS

Strain isolation: The organism was isolated from soil samples were taken from various places including crop fields in Egypt in clean plastic bags and stored at 4°C. 0.2 mL of 10% soil suspension were added to 50 mL of the media in 250 mL flasks which prepared according to Bender and Wallenfels (1961) contained (g L^{-1}): Peptone, 8; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; KCl, 0.5; $NaNO_3$, 5. Glycogen was added (0.5%) to the medium for isolation, whereas of starch was added (0.5%) to the medium for purification, for the lower expenses of starch. Media were autoclaved at 121°C for 20 min. Final pH was adjusted at 5.0 using HCl. Culture was incubated at 30°C under shaking conditions at 120 rpm for 24 h. Isolation was done by agar streaking technique and incubated at 30°C for 4 days. Finally, one isolate was selected according to the largest zone for further investigation.

Characterization of organism: The morphological and taxonomic characteristics were examined according to the method of Mac Faddin (1976, 1985) and Sneath (1986).

Enzyme production: A series of 250 mL Erlenmeyer flasks each containing 50 mL of the liquid medium were

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inoculated with 1 mL of 18 h broth culture of the organism and incubated for 3 days at 30°C under shaking condition. Crude enzyme preparation was obtained from the supernatant after centrifugation of the broth culture at 5000 g in sigma cooling centrifuge for 20 min.

Assay of isoamylase: Isoamylase activity was assayed according to modified method from Maruo and Kobayashi (1951). The reaction mixture contained 0.5 mL amylopectin 2% (w/v) as substrate in acetate buffer at ph 5.0 and 0.5 mL enzyme solution. After incubation at 40°C for 1 h, 0.2 mL of the reaction mixture was with drown and mixed with 2.0 mL of iodine solution (0.005% I_2 and 0.015% KI). Water was then added to a total volume of 8 mL. Activity was monitored by measuring absorbance at 620 nm in a spectrophometer (UV-Visible 240 IPC shimadzu , Kyoto, Japan).

Enzyme unit of isoamylase activity: (U) was defined as an increment in absorbency at 620 nm of 0.01 in 60 min at 40°C under assay conditions.

Protein determination: Protein was determined by Bradford method (Bollag and Edelstein, 1991) using bovine serum albumin as standard to calculate the specific activity.

Effect of incubation period, temperature and pH on enzyme production: The effect of incubation period, temperature and pH on isoamylase production was investigated by cultivating the organism at: different incubation time (6 -144) h and different temperatures (20-37°C) for obtaining the optimum time and at pH (3.6-7.6). The organism was incubated, the isoamylase activity and protein were determined in supernatant.

Inoculum size, carbon and nitrogen sources: Various inocula sizes (25-200) µL per 50 mL medium broth 24 h old were inoculated into the media, and incubated at 30°C for 72 h. Three major of carbon sources, starch, amylopectin and glycogen were tested at 1% (w/v) concentration. Nitrogen sources: peptone, urea, (NH_4) SO_4, NH_4Cl and ammonium molybdate at concentration 1%.

RESULTS AND DISCUSSION

Characterization of organism: The isolated organism was identified as *Bacillus coagulans* as shown in Fig. 1 and Table 1 according to morphological and biocmical testes and in comparison with Sneath (1986).

Enzyme production and assay: Although, it was not reported before to produce isoamylase, *Bacillus coagulans*, isolated from Egyptian soil, showed good

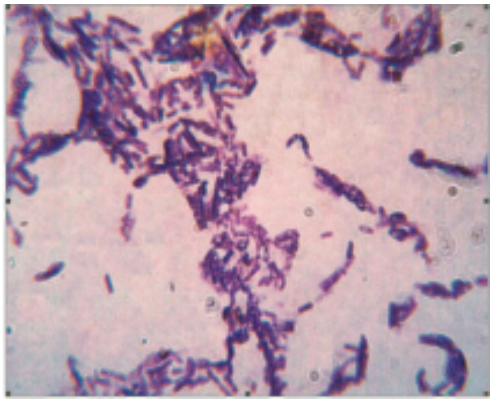

Fig. 1: A photomicrograph of gram's stain of the identified bacterial *B. coagulans* CF_2

Table 1: Identification of acidotolerant strain

Character	CF_2	Bergey's1986
Morphological and microscopic character		
Colony color	Creamy buff	ND
Cell form	Short rods	Rods
Motility	Motile	+
Gram's stain	+	+
Spore formation	+	+
Nitrate reduction to nitrite	+	d
Gas from nitrate	-	-
Productivity		
Amylase	+	d
Catalase	+	+
Caseinase	+	d
DNase	+	ND
Gelatinase	+	d
Oxidase	+	ND
Urease	+	-
Indole production	-	-
H_2S production	-	-
L-Lycine decarboxylase	-	ND
L-Lycine deaminase	-	ND
Tolerance of high NaCl concentration (%)		
2	+	ND
5	+	-
7	+	-
10	-	-
D (-) Arabinose	-	-
Aesculine utillization	±	ND
Cellobiose	+	ND
D (+) Galactose	+	ND
D-Glucose	+	d
α-Lactose	+	ND
Minnitol	+	d
D+ Mannose	+	ND
D-(+)-Xylose	-	-
Gas from glucose	-	-
Growth at different temperatures (°C)		
5	-	-
10	-	-
20	+	ND
30	+	d
40	+	+
50	+	d
55	-	d
65	-	-

Table 1: Continue

Character	CF_2	Bergey's1986
Growht at different pH values		
3.6	ND	ND
4.6	+	ND
5.6	+	d
6.6	+	+
7.6	+	ND
8.6	+	ND
9.6	-`	ND
10.6	-	ND
Carbohydrates fermentation		
L-LYcine deaminase	-	ND
Oxidation / Fermentation	Oxidative	ND
Ornithine decarboxylase	-	ND
V.P test	±	-
Blood hemolysis	α-hemolysis	ND
Growth on MacConkey agar	-	ND
Citrate utilization	-	d
Methyl red test	+	ND
Growth under anaerobic conditions	-	-

D: Different reactions in different taxa, d: 11-89% of strains are positive, ND: No data available

Table 2: The effect of incubation period on *B. coagulans* isoamylase productivity*

Incubation period (h)	Isoamylase productivity (U mL^{-1})	Total protein (μg mL^{-1})	Specific activity (U μg^{-1})
6	11.33	41.07	0.274
12	25.33	48.93	0.518
24	31.33	55.17	0.568
48	46.33	56.57	0.819
72	56.67	59.94	0.945
96	60.33	67.87	0.889
120	80.33	62.33	1.225
144	38.67	57.67	0.670

*The test was carried out at pH 5, temp. 30°C and under shaking conditions. Starch was added to the medium as carbon source (0.75 %), $(NH_4)H_2PO_4$ as nitrogen source and inocula size (100 L 50 mL^{-1} medium)

Table 3: The effect of different incubation temperatures on *B. coagulans* isoamylase productivity*

Temperature (°C)	Isoamylase productivity (U mL^{-1})	Total protein (μg mL^{-1})	Specific activity (U μg^{-1})
20	0.00	18.35	0.000
25	11.67	27.61	0.423
30	79.00	60.53	1.305
37	8.33	42.88	0.194

*The test was carried out at pH 5 under shaking conditions for 5 days. Starch was added to the medium as carbon source (0.75 %), $(NH_4)H_2PO_4$ as nitrogen source and inocula size (100 μL 50 mL^{-1} medium)

isoamylase productivity compared with other isolates using Maruo and Kobayashi (1951) as a widely used assay method.

Optimization of isoamylase production by *Bacillus coagulans*:

Effect of incubation period: The time course from 6 to 144 h was followed in liquid shaking culture medium at 30°C and initial pH 5. Table 2 showed the effect of different incubation periods on *B. coagulans* isoamylase productivity and also showns that 120 h or 5 days was the optimum incubation period for isoamylase production. It is note-worth that the increase in isoamylase productivity synchronizes with the start of death phase of the organism *B. coagulans*. We noted that the maximum isoamylase activity is synchronizing with the decrease in total protein content which needs further illustrations in the following works.

Effect of the temperature: The results in Table 3 indicated that the maximum production of isoamylase enzyme in the temperature at 30°C. At 37°C, the activity decreases 89.5% than the activity at 30°C while the activity was completely inhibited at 20°C.

Effect of pH: Results recorded in Table 4 indicates that *B. coagulans* grows well at pH range of (4.6-7.6), weakly at pH (8.6) and failed to grow over pH (8.6). Therefore, *B. coagulans* isoamylase productivity was assayed between pH (3.6-7.6). Table 4 represented isoamylase activity and total protein for the organism *B. coagulans* at pH (3.6, 4.6, 5.6, 6.6 and 7.6). Results indicate that the use of buffers for the adjustment of the medium pH for isoamylase production is inconvenient. Also the results in Table 4 indicated that the maximum production of isoamylase enzyme was at pH 5.6 by using amylopectin followed by glycogen and the lowest productivity was by using starch as carbon source.

Table 4: Effect of different pH values on *B. coagulans* isoamylase productivity using different carbon sources*

	Carbon sources					
	Starch		Glycogen		Amylopectin	
pH	Isoamylase productivity (μ mL^{-1})	Total protein productivity (μg mL^{-1})	Isoamylase (μ mL^{-1})	Total protein (μ mL^{-1})	Isoamylase (μ mL^{-1})	Total protein (μ mL^{-1})
Control	12.00	57.56	7.50	107.42	10.67	105.03
3.6	0.00	98.63	0.00	75.85	0.00	84.35
4.6	0.00	122.28	0.10	99.21	0.00	95.25
5.0	0.00	128.45	1.67	104.97	1.33	102.59
5.6	0.67	173.60	1.67	109.17	2.67	114.70
6.6	1.33	114.53	3.33	77.60	0.67	112.78
7.6	0.00	93.67	0.00	93.62	0.00	89.71

*The medium pH was adjusted using buffers; Citrate-phosphate buffer (3.6-6.6) and Phosphate buffer (7.6). Control: Original medium adjusted at pH 5 using HCl

Table 5: Effect of different inoculum sizes on *B. coagulans* isoamylase production*

Inoculum size (µL 50 mL^{-1} medium)	Isoamylase activity (U mL^{-1})	Total protein (µg mL^{-1})	Specific activity (U µg^{-1})
25	15.00	75.32	0.198
50	38.67	73.28	0.528
100	53.33	59.25	0.900
125	43.33	60.29	0.719
150	43.67	63.09	0.692
200	24.67	50.22	0.491

*The test was carried out at pH 5, temp. 30°C and under shaking conditions for 72 (h). Starch was added to the medium as carbon source (0.75 %) and (NH4)H_2PO_4 as nitrogen source

Table 6: The effect of different starch concentrations on *B. coagulans* isoamylase productivity*

Starch concentration (%)	Isoamylase productivity (U mL^{-1})	Total protein (µg mL^{-1})	Specific activity (U µg^{-1})
0	0.00	38.51	0.000
0.25	13.67	60.82	0.225
0.50	31.00	76.02	0.408
0.75	43.33	62.97	0.688
1.00	26.67	71.36	0.374
2.00	6.33	51.03	0.124

*The test was carried out at pH 5, temp. 30°C and under shaking conditions for 72 (h). The test was carried out with $(NH_4)H_2PO_4$ as a sole nitrogen source

Table 7: The effect of different nitrogen sources on *B. coagulans* isoamylase productivity

Nitrogen source	Nitrogen source (%)	Isoamylase productivity (U mL^{-1})	Total protein (µg mL^{-1})	Specific activity (µµg^{-1})
Control*	0.201	11.67	56.00	0.207
Peptone	1.355	0.00	114.18	0.000
Ammonium molybdate	2.960	0.00	103.93	0.000
Ammonium citrate	1.803	6.33	79.93	0.079
Urea	0.431	26.00	151.23	0.172
$(NH_4)_2SO_4$	0.948	0.00	92.04	0.000
$(NH_4)H_2PO_4$	1.653	31.67	74.10	0.427
$(NH_4)_2HPO_4$	0.949	6.00	98.92	0.061
NH_4Cl	0.769	13.33	120.18	0.111
NH_4NO_3	0.575	9.00	2.56	3.511
$NaNO_3$	1.221	0.50	40.89	0.012

*Control: Original medium containing peptone and $NaNO_3$. The test was carried out at pH 5, temp. 30°C and under shaking conditions for 72 h. Starch was used as the carbon source. Nitrogen sources were added in equal nitrogen content.

Effect of inoculum size: The effect of inoculum size on the production of isoamylase was shown in Table 5 indicated that the inoculum size at 100 µL /50 mL medium affected the enzyme production. Even the important of inoculum size as a parameter controlling of the production of enzyme (Abd El-Rahman, 2006) most scientists on isoamylase production did not take it in consideration.

Effect of carbon sources: Three carbon sources (starch, amylopectin and glycogon) were used in this study at 1% w/v in production medium. Starch and amylopectin at the concentration 1% proved to be the best inducer for isoamylase production (10 and 10 U mL^{-1}) compared with glycogen 4 U mL^{-1}. Although starch showed the best specific activity (0.180 U µg^{-1}) compared with amyolpectin (0.097 U µg^{-1}). Data showed in Table 6 indicated that the 0.75% (w/v) is the optimum soluble starch concentration for isoamylase production (43.33 U mL^{-1}).

Effect of nitrogen source: Table 7 shows that $(NH_4)H_2PO_4$ was the best nitrogen source that produces maximum isoamylase activity (31.67 U mL^{-1}) while peptone, $(NH_4)_2SO_4$ and ammonium molybdate were inhibited the enzyme production.

CONCLUSION

Bacillus coagulans isolated from the Egyptian soil showed good isoamylase productivity compared with other isolates. The optimization of the nutritional and environmental conditions for *Bacillus coagulans* isoamylase production revealed that pH 5, temperature 30°C aeration and inoculum size 100 µL /50 mL medium for 120 h were the optimum environmental conditions, while 0.75% (w/v) starch, 1.078% (w/v) glycine were the optimum nutritional conditions for the enzyme production.

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