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INFLUENCE OF MEDIA COMPOSITION ON THE PRODUCTION OF ALKALINE α-AMYLASE FROM *BACILLUS SUBTILIS* CB-18

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ABSTRACT

Background. Starch, a homopolysaccharide is an important and an abundant food reserve and energy source. Starches are processed to yield different products which find many industrial applications. Alpha-amylases hydrolyze starch by cleaving α-1,4-glucosidic bonds and have been used in food, textile and pharmaceutical industries [Sun et al. 2010]. Enzymatic conversion of starch with amylase presents an economically superior alternative to the conventional method of starch gelatinization. Alkaline α-amylase has an important position in the global enzyme market as a constituent of detergent. In this paper, we screened soil bacteria and an isolate, alkalophilic *Bacillus subtilis* CB-18 was found to produce an alkaline α-amylase in different media.
Material and methods. Screening of the isolates for amylolytic activity was carried out by growing bacteria isolated from the soil in starch agar plates and subsequently staining the plates with iodine solution to reveal zones of hydrolysis of starch. The selected isolate, *Bacillus subtlis* CB-18 was grown in different media at alkaline pH to evaluate the influence of media composition on alkaline α-amylase production. Enzyme assay was carried out by growing the culture in a broth medium and obtaining cell-free culture supernatant after centrifugation at 2515 × g for 15 minutes. Amylase activity was determined by incubating 0.5 ml of crude enzyme solution in 0.1M Tris/HCl buffer (pH 8.5) with 0.5 ml of 1% soluble starch solution. The reaction was terminated by the addition of DNS reagent and reducing sugar produced from the amylolytic reaction was determined.
Results. *Bacillus subtilis* CB-18 used for this work was selected because it produced 7 mm zone diameter on starch agar plate. This organism was cultured in different alkaline broth media containing 2% soluble starch as inducer carbohydrate for α-amylase production. Among the carbon sources used for enzyme production, sorbitol was the best to stimulate enzyme production with α-amylase activity of 758 U/mL after 48 h. Peptone was the best nitrogen source for enzyme production with α-amylase activity of 680 U/mL after 48 h. Metal ions including Ca^{2+}, Mn^{2+} and Mg^{2+} stimulated enzyme production while Hg^{2+} and Ag^{+} repressed enzyme production. The best enzyme yields were observed in basal media containing agro-based substrates.
Conclusion. This work reports the production of alkaline α-amylase by *Bacillus subtlis* CB-18 in different media. Enzyme production was highest when agro-based media were used to formulate the media.

Key words: alkaline α-amylase, *Bacillus subtilis* CB-18, agro-based substrates

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INTRODUCTION

Many bacteria have the ability to synthesize a variety of extracellular enzymes and most of these bacteria have been isolated from the soil. Alpha amylases (α-1,4-alpha-D-glucan glucanohydrolase, EC 3.2.1.1) are enzymes which hydrolyze α-1,4-glucosidic bonds in starch molecules to products like dextrin and progressively smaller compounds of glucose units. Alpha amylase acts by splitting the bonds in the interior of starch with the production of reducing sugars. Alpha amylase is a specific enzyme acting only at the 1,4-α-bonds and by – passes 1,6-α-bonds. The initial stage of action of this enzyme is characterized by rapid change of its iodine staining property and reduces the viscosity of dextrin of varying chain length [Priest 1992]. Amylases are one of the most important industrial enzymes which can be used in a number of industrial processes including brewing, baking, textiles and detergents [Gupta et al. 2003]. Enzymes capable of catalyzing the hydrolysis of starch are widely distributed in nature and are produced by a variety of members of the genus *Bacillus* [Kim et al. 1996, Lu et al. 2010]. Certain species of bacteria grow in alkaline media and produce large amounts of alkaline amylases [Horikoshi 1996]. The application of such enzyme requires its stability and usage at alkaline pH.

Amylase enzymes have a wide variety of applications in the food and other biotechnological industries. In the food industries, amylases are used for the production of sugar syrup [Van der Veen et al. 2004] and starch processing [Poonam and Dalel 1995]. Conversion of starch into sugar syrups (glucose, maltose, maltotriose, or fructose syrups etc.) forms the major part of the starch processing industry. The hydrolysates of starch are used as carbon sources in fermentation as well as sources of sweetness in a range of manufactured food products. Alkaline α-amylases can be useful in related applications. Alkaline amylases also retain activity at the pH at which detergents function [Ito et al. 1998]. This work reports the influence of media composition on alkaline amylase production from *Bacillus subtilis* CB-18 isolated from the soil.

MATERIAL AND METHODS

Sample collection

Soil sample was collected from a refuse dump near the University campus into sterile polyethylene bag. The sample was sieved through a 1mm sterile mesh to remove coarse materials. Soil sample (10 g) was dissolved in 90 mL of distilled water (pH = 7.6) contained in 500 ml Erlenmeyer flask. The pH of the sample was adjusted to 8.5 using 0.2 M NaOH and the sample was incubated in a Gallenkamp orbital incubator for 24 h at 100 × g. After incubation, l mL of the solution was withdrawn with a sterile pipette and serially diluted in 9 mL 0.1% peptone water diluents. The diluted sample was plated onto starch (Merck, Darmstadt) agar plates and incubated at 30°C for 24 h. Pure cultures were assigned arbitrary numbers and stored at 4°C. The isolated strains were streaked on starch (Merck, Darmstadt) agar plates and incubated at room temperature for 72 h. After incubation, 1% iodine solution was layered on the plates and zones of clearing around the colonies were measured. The isolate designated CB 18 was selected for further work because it produced the best hydrolysis of starch agar. The isolate was identified as *Bacillus subtilis* based on the API 50 CHB identification system (BioMerieux, Marcy-L' Etoile, France) in combination with the APILAB software. Further morphological and physiological tests as outlined in Bergey's Manual of Determinative Bacteriology [Holt et al. 1994] were also used to identify the isolate.

Medium

The medium for microbial cultivation designated Medium A contained the following: 2% soluble starch (Merck, Darmstadt); 0.5% peptone (Oxoid), 0.2% Na_2HPO_4 and 0.1% KH_2PO_4. The final pH was adjusted to 8.5 using 0.2 M NaOH. The medium was sterilized at 121°C for 15 minutes. The inoculum was grown for 24 h in a Gallenkamp orbital incubator at 100 × g at 30°C. The cells were collected by centrifugation using Gallenkamp Junior centrifuge at 2515 × g for 15 minutes, washed twice with 0.1 M Tris/HCl buffer (pH 8.5) and diluted to an optical density of 0.1 measured in a Spectrumlab 23A spectrophotometer at 600 nm. All media (100 mL) contained in 500 mL conical flasks were inoculated with 1 mL of this standard suspension and incubated in a Gallenkamp orbital incubator at 100 × g.

Preparation of locally formulated media

Corn Steep Liquor (CSL): Corn kernels (1 kg) were washed with deionized water and steeped for 4 days in 3 L deionized water and then milled. The slurry was collected in a plastic bucket, stirred and allowed to settle at room temperature (30°C) for 6 h. The clear supernatant was decanted and filtered with a Whatman number 1 filter paper. The pH of the supernatant was adjusted to 8.5 using 0.2 M NaOH solution. The medium was sterilized by tyndallization according to Collins and Lyne [1976].

Tomato Juice Broth (TJB). Fresh tomato fruits (200 g) were homogenized in a blender containing 1 L of deionized water and filtered with a stainless steel mesh. The filtrate was re-filtered with a Whatman No. 1 filter paper and adjusted to pH 8.5 with 0.2 M NaOH. The medium was sterilized by tyndallization.

Orange Broth (OB). Peeled fully-ripped oranges (200 g) were ground with a Corona mill (Medellin, Colombia) after removing the seeds in 1 L of deionized water and filtered with a stainless steel mesh. The filtrate was re-filtered with a Whatman No. 1 and adjusted to pH 8.5 with 0.2 M NaOH. The medium was sterilized by tyndallization.

Banana Broth (BB). Peeled banana fruit (200 g) was homogenized with mortar and pestle in 1 L of deionized water and filtered with a stainless steel mesh. The filtrate was re-filtered with a Whatman No. 1 filter and adjusted to pH 8.5 with 0.2 M NaOH. The medium was sterilized by tyndallization.

Assay procedures

The pH was determined using a glass electrode pH meter (PYE Unicam, England). Protein content was estimated by the method of Lowry et al. [1951] using bovine serum albumin (Sigma-Aldrich) as a standard. Reducing sugar was determined by the dinitrosalicylic acid (DNS) method of Miller [1959] using 50-200 μg glucose as standard.

Amylase activity was assayed by incubating the enzyme solution (0.5 ml) with 1% soluble starch (0.5 ml) in 0.1 M Tris/HCl buffer (pH 8.5). After 30 minutes, the reaction was stopped by the addition of 4 ml DNS reagent then heated for 10 min in boiling water bath and cooled in a refrigerator. Absorbance readings were used to estimate the units of enzyme activity from glucose standard curve. One unit of activity was defined as the amount of enzyme that released 1 μg of glucose from starch per minute under the assay condition.

Determination of end products of starch hydrolysis

Analysis of the end products of starch hydrolysis by the alkaline α-amylase was carried out in a reaction mixture containing 2 ml of 10% soluble starch (Merck, Darmstadt) and 2 ml crude enzyme solution in 1 ml 0.1 M Tris/HCl buffer (pH 8.5) contained in a test tube. Samples were taken at 5 h intervals and analyzed by HPLC using an NH_2-18C column (Merck, Darmstadt). The column was maintained at 38°C with 80% (v/v) acetonitrile in HPLC grade deionized water as the mobile phase. Elution was done at a flow rate of 1.0 $ml \cdot min^{-1}$. Sugar standards used were glucose, maltose and maltotriose.

The effects of carbon sources on alkaline α-amylase production was determined by incorporating various carbon sources – xylose (BDH), arabinose (BDH), glucose (BDH), fructose (BDH), maltose (BDH), lactose (BDH), raffinose (Sigma-Aldrich), mannitol (BDH), sorbitol (Difco) and sucrose (Matheson Coleman and Bell) at 1% (w/v) concentration into medium A.

To investigate the effects of nitrogen sources on alkaline α-amylase production, 0.5% (w/v) of various nitrogen sources namely, yeast extract (Difco), malt extract (Difco), tryptone (Oxoid), urea (BDH), ammonium sulphate (BDH), sodium nitrate (BDH), potassium nitrate (May and Baker) were added into medium A in place of peptone.

To test the effects of metal ions and reagents on enzyme activity, the enzyme was dialyzed overnight against 0.1 M Tris/HCL buffer (pH 8.5). This enzyme solution was used to study the metal ion requirement for enzyme activity. The metal ions and reagents were added separately into Medium A and the reducing sugar formed was assayed.

Statistical analysis

The significance of the tests was evaluated by the analysis of variance (ANOVA).

RESULTS AND DISCUSSION

The isolated microorganisms were tested for their ability to qualitatively hydrolyze starch agar plates

(Table 1). The isolate designated CB-18 produced 7 mm zone of clearing on starch agar after 72 h incubation and was therefore selected for further studies. Yetti et al. [2000] reported a widest zone of hydrolysis of starch agar of 5 mm for *Acremonium* sp. The selected microorganism was later identified as *Bacillus subtilis* based on its morphological and some physiological characteristics. Many researchers have reported the use of *Bacillus subtilis* for industrial production of alkaline α-amylase [Sivaramakrishnan et al. 2006, Sarety et al. 2011].

The predominance of glucose in the hydrolysate (Fig. 1) suggests that the α-amylase from *Bacillus subtilis* CB-18 is a saccharifying type. Similar amylase has been reported in *Bacillus subtilis* [Nagata et al. 1980]. Liquefying enzyme which occurred in *Bacillus subtilis* KCC103 produced predominantly maltooligosaccharides during starch hydrolysis [Nigarajan et al. 2008].

Table 1. Selection of isolates based on their ability to hydrolyze starch

Isolate	Zone of clearing, mm
CB 1	2
CB 2	0
CB 3	5
CB 4	1
CB 5	3
CB 6	3
CB 7	1
CB 8	3
CB 9	0
CB 10	4
CB 11	3
CB 12	2
CB 13	1
CB 14	2
CB 15	5
CB 16	3
CB 17	1
CB 18	7

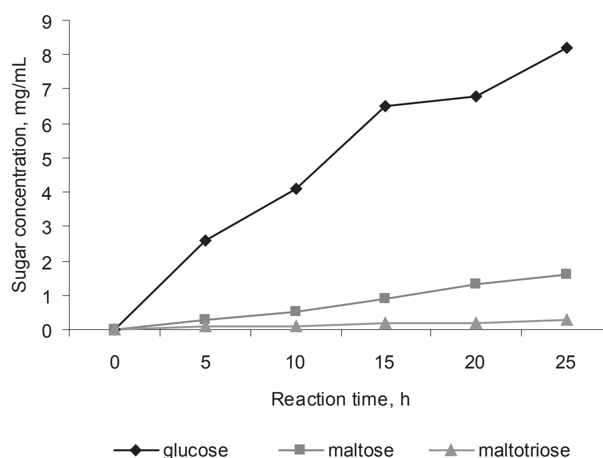

Fig. 1. Products of hydrolysis of soluble starch by alkaline α-amylase enzyme from *Bacillus subtilis* CB-18

Different pure carbon sources including xylose, arabinose, glucose, fructose maltose, lactose, raffinose, mannitol, sorbitol and sucrose were employed individually into the basal medium used for alkaline α-amylase synthesis by *Bacillus subtilis* CB-18 at 1% (w/v) concentration (Table 2). Soluble starch was used in the medium as the inducer carbohydrate for enzyme production [Upton and Fogarty 1977] and was incorporated into the medium at 2% concentration. Among all the pure carbon sources used in the basal medium for enzyme synthesis, sorbitol was found to be the best carbon source for alkaline α-amylase production by the organism. Alpha amylase unit of 758 U/mL was produced after 48 h and this level decreased to 469 U/mL after 72 h. There was a significant ($p \leq 0.05$) difference in the use of various carbon compounds for enzyme production (Table 2). In general, best enzyme production for all the tested carbon sources occurred after 48 h. This may be associated with the onset of the stationary phase of microbial growth during which microbial metabolite production is highest [Kelly et al. 1984]. Culturing of the organism in the presence of glucose repressed enzyme synthesis. Similar effects of glucose on the production of microbial α-amylase enzymes have been reported [Heinken and O'Connor 1972].

The effects of different nitrogen sources on α-amylase production is shown in Table 3. The best nitrogen source which induced enzyme production was peptone (680 U/mL) while enzyme production was lowest when

Table 2. Effects of carbon sources on the production of alkaline α-amylase enzyme by *Bacillus subtilis* CB-18

Carbon source 1% w/v	Alkaline α-amylase activity, units/mL		
	period of incubation, h		
	24	48	72
Control*	317	498	276
Xylose	580	721	429
Arabinose	511	690	321
Glucose	209	287	126
Fructose	416	485	342
Maltose	218	411	179
Lactose	318	529	302
Raffinose	412	592	388
Mannitol	480	609	472
Sorbitol	692	758	469
Sucrose	403	477	216

*Control – medium without additional carbon source.
Values are significantly different ($p \leq 0.05$).

Table 3. Effects of nitrogen sources on the production of alkaline α-amylase enzyme by *Bacillus subtilis* CB-18

Nitrogen source 0.5% w/v	Alkaline α-amylase activity, units/mL		
	period of incubation, h		
	24	48	72
Control*	106	172	89
Peptone	422	680	309
Yeast extract	360	611	296
Malt extract	401	599	311
Tryptone	486	696	318
Urea	211	216	109
Ammonium sulphate	265	344	216
Sodium nitrate	260	296	211
Potassium nitrate	127	209	132

*Control – medium without added nitrogen.
Values are significantly different ($p \leq 0.05$).

potassium nitrate was used for enzyme production. Vidal et al. [1995] also reported in their findings that peptone was the best nitrogen source for amylase production. Organic nitrogen sources were generally the better substrates for enzyme production than inorganic nitrogen sources (Table 3). Similar effects on α-amylase production were reported by Hernandez et al. [2006].

The effects of metal ions and surfactants on enzyme activity is shown in Table 3. Amylolytic activity was best activated in the presence of Ca^{2+} (% relative activity = 192%). Alpha-amylases produced by the genus *Bacillus* are metalloenzymes having calcium as a co-factor and the complete removal of calcium from enzymes produced by *Bacillus* species leaves an inactive protein which can be reactivated in full on the restoration of the divalent cation [Fischer and Stein 1960]. Calcium ions are required for amylase production (Kim et al. 1996) but Ca^{2+} – independent α-amylase has been reported [Haki et al. 2008]. Mg^{2+}

Table 4. Effects of various metal salts and surfactants on the production of alkaline α-amylase enzyme by *Bacillus subtilis* CB-18

Metal salt 0.5 mM	Specific activity U/mg protein	% relative activity
None	113.4	100
$MgSO_4 \cdot 7H_2O$	187.2	165
$AgNO_3$	52.0	46
$BaCl_2$	69.6	61
$CaCl_2$	217.5	192
$CuCl_2$	109.2	96
$MnCl_2$	178	156
$FeCl_2$	92.8	82
$HgCl_2$	42.4	37
$ZnSO_4 \cdot 7H_2O$	102.4	90
Surfactants (0.05%)		
Tween-80	122.9	108
Triton X-100	79.0	70
EDTA	72.8	64

and Mn^{2+} also induced enzyme production (% relative activities of 165 and 156% respectively). The metal ions might have played a role in maintaining the active conformations of the enzyme. The enzyme also retained activity in the presence of Fe^{2+}, Cu^{2+} and Zn^{2+} but Hg^{2+}, Ag^{3+}, SDS and EDTA repressed enzyme synthesis. Amylase enzyme inhibition by mercuric ions, SDS and EDTA has been reported [Arikan et al. 2008].

From this study, corn steep liquor, tomato juice broth, banana broth and orange broth were found to be the best inducers of alkaline α-amylase enzyme production by the bacterium (Table 5). Best alkaline α-amylase activity (844 units/mL) was obtained when corn steep liquor was added into the basal medium used for enzyme production. Formation of a cost-effective medium for microbial enzyme production is of primary interest in view of the commercial applications of microbial enzymes. The use of agro-based products which are locally available and inexpensive substrates for microbial growth and metabolite production has been investigated by many researchers [Gressesse 1997, Johnvesly et al. 2002]. Agricultural by-products and waste products such as corn syrup, wheat bran and cassava waste have been used to support optimal production of α-amylase enzyme [Sun et al. 2010]. Hang and Woodams [1977] reported a very high level of α-amylase and amyloglucosidase production by *Aspergillus foetidus* when grown on baked bean waste as compared with other media. Corn step liquor, a major by-product of the corn wet-milling industry is an inexpensive source of nutrients used for fermentation and for enzyme production [De Azeredo et al. 2006]. Bananas are known to have a number of properties which promote good growth of bacteria [Aegeter and Dunlap 1980]. Tomato juice, an ingredient commonly added to media for culturing bacteria serves as an essential adjunct for the propagation of fastidious bacteria which require growth factors present in tomato juice to initiate growth [Babu et al. 2002]. Orange broth concentrate contains nutrients necessary for the growth of microorganisms [Lequerica and Lafuente 1977].

Table 5. Production of alkaline α-amylase enzyme by *Bacillus subtilis* CB-18 in locally – formulated media

Media	Alkaline α-amylase activity, units/mL		
	period of incubation, h		
	24	48	72
Corn steep liquor	427	844	402
Tomato juice broth	398	603	217
Banana broth	342	596	278
Orange broth	394	788	313

Values are not significantly different ($p > 0.05$).

CONCLUSION

The results presented in this study show the effect of media components on the production of alkaline α-amylase by *Bacillus subtilis* CB-18. Agro-based substrates were comparably the better media than some chemically – defined media for enzyme secretion by the bacterium. Enzyme production using these inexpensive and easily – available agro-based media was therefore recommended.

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