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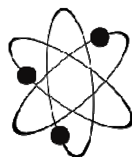

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Production of Alpha Amylase by *Bacillus cereus* in Submerged Fermentation

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Abstract - Microorganisms have the ability to secrete enzymes when they are grown in the presence of certain substrates. Amylases are among the most important industrial enzymes and are of great significance in biotechnological studies. Bacteria belonging to the genus *Bacillus* were isolated using mannitol egg yolk polymyxin B (MYP) agar a highly selective media for *Bacillus cereus* isolation. The isolates were tested for α-amylase production on nutrient agar supplemented with starch and in submerged fermentation. The bacteria isolated and identified (using the Microgen Bacillus identification kit) were all *Bacillus cereus* and SB2 had the largest zone of hydrolysis of 12mm on nutrient agar supplemented with starch as well as the highest enzyme activity of 1.62U/ml. Amylase activity of 2.56U/ml was obtained after 24 hours incubation in submerged fermentation. When amylase enzyme production parameters where optimized, maximum amylase activity was obtained at a pH of 6.5, temperature of 35^{0}C, incubation time of 24 hours and 4% inoculums concentration. *Bacillus cereus* SB2 is a potential isolate for alpha-amylase production with soluble starch as the sole carbon source in submerged fermentation.
Keywords: *Bacillus cereus*; Alpha-amylase; Submerged; Fermentation; Starch.

Introduction

Amylases are starch degrading enzymes that cleave the α- 1-4 glucosidal linkage of complex polysaccharides (Pandey *et al*., 2000). Amylases have potential applications in the food, fermentation, textile, paper and pharmaceutical industries. Amylases have been most widely reported to occur in microorganisms, although they are also found in plants and animals. Currently, they comprise about 30% of the world enzyme production (Vander *et al*., 2002). The main microbial sources for amylase production are *Bacillus* species (Nurmatov *et al*., 2001) and *Asperigillus* species. Considerable information is available on the microbial production of α-amylase in solid media (Ramachandran *et al*., 2004) and liquid media under stationary and submerged conditions (Francis *et al.,* 2003). Two major classes of amylases have been identified in microorganisms, namely alpha-amylase and glucoamylase. Alpha-Amylases (endo-1,4-a-D-glucan glucohydrolase, are extracellular enzymes that randomly cleave the 1,4-a-D-glucosidic linkages between adjacent glucose units in the linear amylose chain. Glucoamylase (exo-1, 4-a-D-glucan glucanohydrolase) hydrolyzes single glucose units from the non-reducing ends of amylose and amylopectin in a stepwise manner (Hema *et al.,* 2006).

Among various extracellular enzymes, alpha-amylase ranks first in terms of commercial exploitation (Babu *et al*., 1993). Spectrum of applications of alpha-amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification, they also find applications in baking, brewing, detergent, textile, paper and distilling industry (Ramachandran *et al*., 2004). Amylases from plant and microbial sources have been employed as food additives. Barley amylases have been used in brewing industry; fungal amylases are widely used for the preparation of oriental foods. In spite of the wide distribution of amylases, microbial sources, namely fungal and bacterial amylases are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production (Parmar and Pandya, 2012).

The amylase producing bacteria such as *Bacillus subtilis, B. licheniformis, B. amyloliquefaciens, B. cereus*, and *B. megaterium* and fungi such as *Aspergillus niger, penicillium, Rhizopus, Cephalosporium and Nuerospora* are major amylase producing microorganisms (Pandey, 2003). Microbial amylases have been proved to be an alternative to chemical hydrolysis and low yield of enzyme has always been a problem in the commercial production of amylases (Monga *et al*., 2011). Therefore, the present work was undertaken to screen various *Bacillus cereus* isolates for α-amylase production and optimization of fermentation conditions for maximum yield.

Materials and Methods

Sample collection and isolation of *Bacillus* species

Soil samples were taken from 5 cm depth after removing 5 cm from the earth surface of refuse dump sites in Zaria which is located at the northern part of Kaduna state, Nigeria. The samples were collected into sterilized plastic bags and transferred to labelled screw-caped bottles. Ten gram (10g) of soil samples were dissolved in 90ml of sterile distilled water and diluted up to 10^{-4} times from which 0.1ml were pipette onto plates of Mannitol Egg-Yolk Polymycin B Agar (MYP) (Oxoid, Hampshire, England), a highly selective medium for *Bacillus cereus* developed by Holbrook and Anderson, 1980. The inoculated agar plates were incubated at 37 ^{o}C for 24 hours. Colonies that appeared pink and were surrounded by whitish zones of clearance on the *Bacillus cereus* selective agar plates were picked and sub-cultured on plates of nutrient agar. The isolates were further transferred to nutrient agar slants, incubated for 24 hours and stored at 4 ^{0}C in the refrigerator until required for use.

Identification of presumptive *Bacillus* species

Bacillus specie were identified based on the cultural, microscopic and biochemical characteristics and confirmed using the microgen *Bacillus* identification kit.

Cultural characterization and microscopic observation

The colonial morphology were noted with respect to shape, colour, size and nature of colony. The isolates were Gram stained and observed under a high power magnifying lens of the light microscope using × 100 oil immersion objectives. Endospore staining was performed to observe the morphology of the cells (Sneath, 1984).

Biochemical characterization

Biochemical tests such as catalase test, Voges-Proskauer test, nitrate reduction test, and lecithinase production tests were carried out on the isolates. The presumptive *Bacillus* spp. were confirmed using the Microgen Bacillus identification kit (Microgen Ltd, UK) (Sneath, 1984).

Screening of isolates for amylase production on nutrient agar plates supplemented with starch.

The isolates were spot inoculated on nutrient agar plates supplemented with 1% soluble starch and incubated at 37^{0}C for 24hours. Clear zones of hydrolysis around the colonies were noted after staining with iodine solution (Ashwini *et al*., 2011)).

Preparation of crude amylase

The production medium contained in g/l, Bacteriological Peptone 6, $MgSO_4.7H_2O$ 0.5, KCl 0.5, soluble starch1g and the pH adjusted to 6.5. A loopful of bacterial culture was inoculated into the medium and cultivated by shaking flask at 200 rpm, for 24 hours at 37°C. Prior to inoculation, 40 ml of medium was distributed into 50 ml Erlenmeyer flasks and sterilized by autoclaving at 121ºC for 15 minutes and allowed to cool down to room temperature (Abe *et al*., 1988).

Amylase assay

Cell-free culture filtrates were used as crude enzyme preparation to assay amylase activity. Amylase activity was determined by the method described by Zambere (2011). The reaction mixture consisted 1.25ml of 1% soluble starch 0.25ml of 0.1M acetate buffer (PH 5.0), 0.25ml of distilled water and 0.25ml of crude enzyme extract. After 10 minutes incubation at 50°c, the liberated reducing sugars (glucose equivalents) were estimated by dinitrosalicylic (DNS) method of

Zambere (2011). The blank contained 0.5ml of 0.1M acetate buffer (PH 5.0), 1.25ml of 1% starch solution and 0.25ml of distilled water. Colour intensity was determined at 540nm using a spectrophotometer. One International Unit (IU) of alpha amylase was defined as the amount of enzyme that releases one milligram of reducing-sugar (glucose equivalents) per minute under standard assay conditions. The isolate with the highest enzyme activity was selected for further studies.

Hydrolysis of starch by *Bacillus cereus*

The investigation of starch hydrolysis by *Bacillus cereus* was carried out in submerged cultivation. The cultivation medium contained in g/l, Bacteriological Peptone 6, $MgSO_4 \cdot 7H_2O$ 0.5, KCI 0.5, soluble starch 1g and the initial pH adjusted to 6.5. Five millilitres (5ml) of bacteria culture that was equivalent to 1.0 McFarland (3.0×10^6 CFU/ml) was inoculated into 250ml of the medium and cultivated by shaking flask at 200 rpm, for 7 days at 37 °C . Supernatants of the fermented broth were collected daily, centrifuged at 5000 rpm for 15 min in a cooling centrifuge and the reducing sugar was determined.

Optimization of the conditions for starch hydrolysis

Incubation time, The effect of incubation time on starch was carried out to determine the optimum incubation time for hydrolysis. The hydrolysis was carried out for 7 days at 37°C and pH of 6.5. Samples were collected daily and analyzed for amylase activity.

*pH***,** The effect of pH on amylase production and starch hydrolysis was determined by culturing the bacterium at various pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9 in the hydrolysis reaction to obtain the optimum pH. Samples were collected after 24hrs for amylase activity determination.

*Temperature***,** Temperature plays an important role in the production of amylase. The effect of temperature was studied by incubating the culture media at various temperatures 30, 35, 40, 45, and 50 °C in order to obtain the optimum temperature for amylase production.

Results and Discussion

Bacillus species are considered to be the most important sources of alpha-amylase and have been used for enzyme production (Pandey *et al.*, 2000). The cultural and physiological properties of the *Bacillus spp* isolated from the soil revealed that all the isolates had colonies that were round in shape. The colonies were raised, large, flat, rough, dry and pink in colour with zones of egg yolk precipitate on MYP agar. The isolates were also rod shaped, gram positive and spore forming indicating they belong to the genus *Bacillus* as shown on Table 1.

The biochemical characterization and identification of *Bacillus cereus* isolates using the Microgen Bacillus-ID System revealed that all the isolates fermented Trehalose and most of them fermented Sucrose. Also, all the isolates were positive to voges proskauer, arginine dihydrolase and nitrate reduction tests (Table 2). They were all confirmed *Bacillus cereus.* The zone of hydrolysis of *Bacillus cereus* on nutrient agar plates that were supplemented with starch showed that SB2 had the largest zone of hydrolysis of 12mm while the reference strain and SB7 had the smallest zones of hydrolysis of 6.0 mm (Figure 1). Also, the amylase activities of the isolates in amylase production media as shown on Table 3 revealed that SB2 also had the highest amylase activity of 1.62U/ml while the reference strain had the lowest amylase activity. The variation in zone of hydrolysis and amylase activities of the isolate could be due to difference in genetic makeup of microorganisms belonging to the same genera and species; it could also be due to the fact that the isolates were obtained from different environments which made them displayed different zones of hydrolysis and amylase activities.

Table 1. Cultural and physiological properties of isolated *Bacillus* specie from soil sample in Zaria

Isolate	Colonial Morphology on MYP Agar	Vegetative Morphology	Gram Reaction	Motility	Starch Hydrolysis	Presence of Spore	Position of Spore	Lecithinase Production
SB1	Rough,dry,bright and pink	Rod Shape	+	+	+	+	Center	+
SB2	Rough,dry,bright and pink	Rod Shape	+	+	+	+	Terminal	+
SB3	Rough,dry,bright and pink	Rod Shape	+	+	+	+	Center	+
SB4	Rough,dry,bright and pink	Rod Shape	+	+	+	+	Center	+
SB5	Rough,dry,bright and pink	Rod Shape	+	+	+	+	Terminal	+
SB6	Rough,dry,bright and pink	Rod Shape	+	+	+	+	Center	+
SB7	Rough,dry,bright and pink	Rod Shape	+	+	+	+	Terminal	+

Note: SH= Soil Bacillus

Table 2. Biochemical characterization of *Bacillus* specie using microgen Bacillus identification kit

Profile Number	Arabinose	Cellobinse	Inositol	Mannitol	Mannose	Raffinose	Rhamnose	Salicin	Sorbitol	Succrose	Trehalose	Xylose	Adonitol	Galactose	Mdm	Mdg	Inulin	Melezitose	ONGP	Indole	Nitrate	Arg	Citrate	Voges Preskauer	Octal Code	Identity
SB1	_	+	_	_	_	_	+	+	_	_	+	_	_	_	_	_	_	+	_	_	+	+	_	+	20620113	*B. cereus*
SB2	_	+	_	_	+	+	_	+	_	+	+	_	_	_	_	_	_	_	_	_	+	+	+	+	23260017	*B. cereus*
SB3	_	+	_	_	_	_	_	+	_	_	+	+	_	_	_	+	_	+	_	_	+	+	_	+	20230513	*B. cereus*
SB4	_	_	_	_	_	_	_	_	_	_	+	_	_	_	_	_	_	_	_	_	+	+	_	+	00020016	*B. cereus*
SB5	_	+	_	_	_	_	_	+	_	+	+	_	_	_	_	_	_	_	_	_	+	+	_	+	20260013	*B. cereus*
SB6	_	+	_	_	+	_	_	_	_	+	+	_	_	_	_	_	_	_	_	_	+	+	+	+	22060017	*B. cereus*
SB7	_	+	_	_	+	_	_	_	_	+	+	_	_	_	_	_	_	_	_	_	+	+	_	+	22060013	*B. cereus*

Nore: Mdm= Methyl-D-Mannoside, Mdg=Methyl-D-Glucoside, Arg= Arginine Dihydrolase,ONGP= O-nitrophenyl-β-D-galactopyranoside, SB=Soil bacillus.

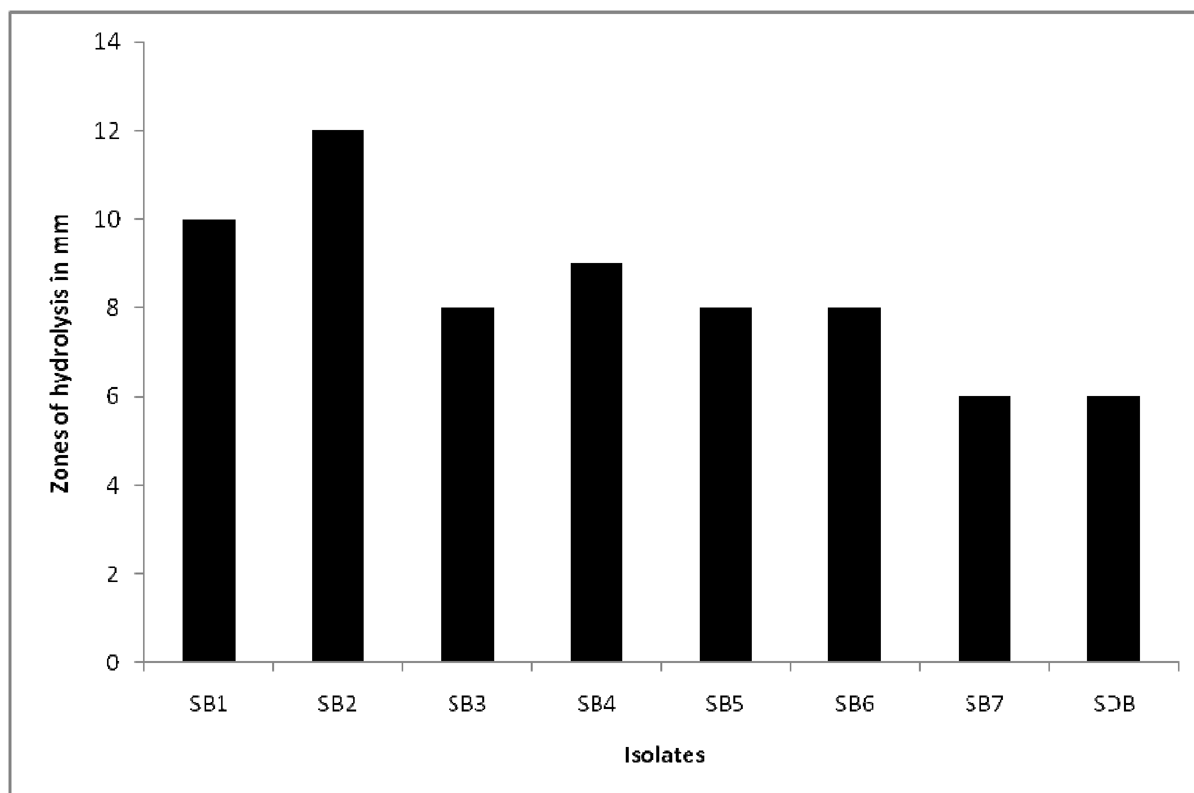

Figure 1. Isolates and their zone of hydrolysis on nutrient agar plates supplemented with starch.

Table 3. Isolates and their amylase activity

Isolate	Amylase activity (U/ml)
SB1	1.46
SB2	1.62
SB3	0.66
SB4	1.25
SB5	0.21
SB6	1.30
SB7	0.65
SDB	0.40

The effect of incubation time on amylase production showed that 24hrs was the optimum duration for maximum amylase activity (Table 4). This might be because; after 24hrs, the cells may have reached the decline phase and displayed low amylase synthesis (Sivakumar *et al.*, 2012). In this study the amylase production by *Bacillus cereus* was found to be maximum at pH 6.5 as shown on Table 4. Further increase in the pH resulted in a decrease in the amylase activity. When pH is altered below or above the optimum, the amylase activity is decreased or becomes denatured ((Vidyalakshmi *et al.*, 2009). Different organisms have different pH optima and decrease or increase in pH on either side of the optimum value results in poor microbial growth (Ramesh and Lonsane, 1991). Ellaiah *et al.* (2002) stated that at high pH, the metabolic activity of bacterium may be suppressed and thus it inhibits enzyme production.

Maximum amylase activity was recorded at 4% inoculums concentration as shown on Table 5. Further increase in inoculums concentration resulted in a decrease in amylase activity. An inoculums concentration higher than the optimum value may produce a high amount of biomass which will rapidly deplete the nutrients necessary for growth and product synthesis. On the other hand lower inoculums levels may give insufficient biomass and allow the growth of undesirable organisms in the production medium (Sivakumar *et al.*, 2012).

The effect of varying incubation temperature on starch hydrolysis by *Bacillus cereus* (SB2) revealed that maximum amylase production was obtained at 35°C (Table 5). Similar optimum temperature (35°C) was observed for the production of amylase from banana stalk using *Bacillus subtilis* as reported Chandrasekaran *et al.* (2012). Further increase in incubation temperature decreased the production of enzyme. Production of the enzyme started reducing at 40°C. This might be due to the fact that at high temperatures, the growth of the bacteria is usually inhibited and hence, enzyme formation is also reduced.

The result of the student t-test carried out (using SPSS version 17) to compare the mean values of the amylase activities of the SB2 under optimized and non-optimized conditions revealed that significant difference exists from day 1 to day 4 at P≤0.05. Under non-optimized conditions (pH 6.5, temperature 37^{0}C, and inoculums size 5%) amylase activity of 2.1mg/ml was obtained after 24hrs incubation whereas under optimized conditions (pH 6.5, temperature 35°C, inoculums size 4%), 1.3 fold amylase activities (2.85 mg/ml) was enhanced when compared with non-optimized conditions indicating the importance of optimization of cultural parameters on amylase synthesis. Maximum amylase activity of 2.56U/ml) was produced by SB2 in this research work which is higher than the amylase activity (1.3U/ml) obtained by Dipali and Argit (2012) when they produced amylase in submerged fermentation using *Bacillus* specie. SB2 is therefore a potential isolate for industrial production of amylase.

Table 4. Effects of incubation time and varying pH on starch hydrolysis

Incubation time (days)	Amylase activity (U/ml)	pH	Amylase activity (U/ml)
1	2.46	6.0	1.06
2	1.58	6.5	1.58
3	0.88	7.0	0.85
4	1.06	7.5	0.58
5	1.23	8.0	0.88
6	1.41	8.5	1.05
7	0.88	9.0	0.89

Table 5. Effects of inoculum concentration and varying temperature on starch hydrolysis

Inoculum concentration (%)	Amylase activity (U/ml)	Temperature (^{o}C)	Amylase activity (U/ml)
2	2.08	30	0.88
4	2.46	35	2.46
6	2.05	40	1.76
8	1.66	45	1.06
10	1.43	50	1.06

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Conclusions

The results obtained in the present study indicated *Bacillus cereus* SB2 as a potential strain for α-amylase production. Since *Bacillus cereus* SB2 has simple nutritional requirements and can produce a good enzyme yield, this isolate could be exploited for α-amylase production in submerged conditions using soluble starch as carbon source.

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