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UTILIZATION OF CORN STARCH AS SUBSTRATE FOR ß-AMYLASE BY BACILLUS SPP

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ABSTRACT

Corn starch was used as substrate for ß -amylase production from ten(10) amylolytic species of the genus Bacillus isolated locally from soil, waste water and food sources. Ten bacillus strains was made up of two strains each of Bacillus macerans, Bacillus licheniformis and Bacillus circulans. Also included are B. coagulans, B. megatarium, B. polymyxa and B. subtilis. B. cereus ATCC 11778 served as type specie. The mean estimate of total bacterial count for all the amylolytic bacillus strains studied was 11.58 X 10^2 CFU/ml in corn starch substrate, 10.1 X 10^2 CFU/ml soluble starch and nutrient broth medium recorded 7.4 X 10^2 CFU/ml. The growth value expressed in corn starch substrates shows its viability as a cheap carbon source. ß-amylase production by the various strains showed that B. licheniformis (S2) (4.2 unit/ml) and B. subtilis (6.24 unit/ml) has high enzymatic activity with corresponding maltose yield of 46.4mg/ml and 68.0mg/ml respectively. Calcium ion added to the assay systems improved ß-amylase activity.

INTRODUCTION

Corn (*Zea mays*) is widely grown in tropical and subtropical countries. Corn products are extensively used for various purposes in the tropics. For example corn-starch is modified for various domestic and industrial purposes as edible food or animal feed. These characteristics stimulate the idea of considering the viability of corn-starch substrate for the production of ß. Amylase from amylolytic *Bacillus* Spp. Amylases represent a group of enzyme of great importance to the food industry and other needs of life. They were also one of the first to be produced industrially by microorganisms (Reed, 1975).

The substrate for amylases is starch. According to Rose (1980), starch occurs in the form of water-insoluble granules as the major reserve carbohydrate in all higher plants. It is produced commercially from the seeds, tubers and roots of plants. The major source of starch is corn, from which it is extracted by a wet milling process. Corn starch can be obtained as a cheap carbon source forming an heterogeneous polysaccharide composed of two high molecular weight components amylose and amylopectin. The two differ significantly in many physical properties notably molecular, size, solubility in water, iodine-staining capacity and susceptibity to enzymic hydrolysis (Rose 1980).

Enzymes responsible for the breakdown of starch are widely distributed in nature. Among these are the

amylases, which act on starch, glycogen and derived polysaccharides to hydrolyze the a - 1, 4- glycosidic linkages. The amylase may thus be divided into three groups: the a - amylases (endoamylases), ß-amylases (exo-amylases) and glucoamylases. The substrate and culture media component greatly influence the nature of amylase enzyme produced (Srivastava and Baruah, 1986). In starch processing industries, immobilized cells were used to optimally exploit the amylase producing machinery of the cells of which the ß - amylase-producing cells are employed for bioconversion of starch to maltose (Ray *et al* . 1995).

There are wide ranges of sporulating-catalase positive, gram-positive rods that belong to the genus *Bacillus* . Many of them have the ability to produce variety of enzymes based on the conditions they are subjected to. The property of some of them to produce amylase that hydrolyzes starch substrate categorized them as the amyloytic *Bacillus* Spp. The action of various amylases on starch granules indicated that starches from various botanical sources display differences in susceptibility towards amylases as demonstrated in previous studies (Reed, 1975). This work was embarked upon to formulate corn-starch substrate for ß- amylase production as revealed in this study.

MATERIALS AND METHODS

The Bacillus spp. Studied for their ß-amylase production, B. *macerans* . B *coagulans* , B. *licheniformis* , B. *circulans,* B. *magaterium,* B . *polymyxa,* and B. *subtilis* were isolated from soil, waste water and food sources in Ibadan and identified by standard microbiological methods. The B. *cereus,* ATCC 11778 served as a type species. The isolates were maintained on nutrients agar the slants at 4oc in the refrigerator for use in the present investigation.

Preparation of substrate:

The starch from white corn was obtained by wet milling process. The soluble corn-starch was sieved with sterile finely galvanized cloth and dried for preservation and further usages.

The work was performed in two phases. In the initial stage 1% each of soluble and corn starch were introduced into a 100ml nutrient broth medium. In the later study, a mineral-salt medium prescribed by Takasaki : (1976) for ß- amylase production in Bacillus spp. was used. The composition of the medium used was 2% peptone, 0.5% soluble starch, 0.3% K_2HP0_4 and 0.1 $MgSO_4.7H_20$. Corn-starch substrate was used as substitute to serve as carbon source during the study.

Effect of Metal ion:-

4mg each, of Ca^{2+} ($CaCl_2H_2O$) and Na+ (NaCl) salts was mixed with one milliliter of enzyme solution for activity and assayed.

Enzyme Assay:

Assay system for amylase activity was carried out by measuring the amount of reducing sugar according to the DNSA method (Murao *et al.,* 1979). The substrate was 1.0% soluble starch dissolved in phosphate buffer (pH 7.0).

One-tenth (0.1) ml of the test solution was added to 1ml of the substrate. After incubation for 10mins at 37oc the reaction mixture was stopped by adding 2ml of DNSA reagent (Murao *et al.* 1979). The reaction mixture was heated at 100oc for 10min and cooled. Then 17ml of water was added to the solution. After allowing the reaction mixture to stand for 15min at room temperature, the optical density was measured at 530.n.m. One unit of Amylase was defined as the amount of enzyme which liberated 1um mole of maltose per min in this assay system (Murao *et al,* 1979 and Bailey, 1988).

Since there is probability of having some amounts of alpha amylase produced together with beta amylase by some *Bacillus* spp., the ratio of the amount of alpha amylase present in most instances was therefore determined by first getting the enzyme solution heated in a water bath at 70℃ for 15 minutes in order to inactivated the beta amylase (Bernfeld, 1951;) and incubate with 1ml of the 1% soluble starch solution. The enzyme assay was equally performed as described above.

Assessment of Corn Starch Substrate as a Cheap Carbon Source for Amylolytic Bacillus Spp.:

The growth pattern and utilization of corn starch as a cheap carbon substrate by the amylolytic *Bacillus*

species compared to soluble starch and nutrient broth medium was determined (Nortemann, 1992).

One ml of each sample cultured for 24hrs, were incubated using a pour plate technique. Total bacterial count of strains cultivated on each of the growth medium mentioned above was enumerated for comparative analysis.

Cultivation of Amylolytic *Bacillus* Spp. for ß - Amaylase Production:

Amylolytic Bacillus. Spp. Studied were cultured in a medium (50ml), containing 2% peptone, 0.5% soluble starch, 0.3% K_2HPO_4 and 0.1% $MgSO_4 . 7H_2O$ in Erlenmeyer flask of 200ml capacity. The cultivation was carried out for about 40hours at 30℃ on a rota tory shaker at 150 rpm. The cultured cell was removed by centrifugation at 4000 rpm for 15 mins. and resultant supernatant was used as the enzyme source. Corn-starch substrate was used interchangeably with soluble starch during the study as one of the basis on which the research is formulated being a common and cheaper carbon substrate compared with others. Starch exists in various forms. Sanni *et al.* (1992) reported the use of cassava peel for amylases production in *Aspergillus sp* . But cassava contains high levels of cynogenic glycosides (Wood, 1965). Corn-starch has no such disadvantages, moreover is cheaply cultivated in Nigeria and several parts of Africa .

RESULTS

Suitability of corn starch substrate for ß- amylase production from Amylolytic *Bacillus* spp.:

Ten amylolytic *Bacillus* species from soil, waste-water and food sources were finally selected for the purpose of this study. *B. cereus* ATCC 11778 served as a typed species. The growth pattern and utilization of corn starch as a cheap carbon substrate by the amylolytic *Bacillus spp.* was determined as shown in Tables 1 to 3. The result revealed that corn starch was actively utilized by the amylolytic *Bacillus* species studied. For example a strain of *B. macerans* (S1) showed a total bacterial count of 20, 15, and 0.6 x 10^2 cfu/ml in corn starch substrate, soluble starch substrate and nutrient broth medium respectively. Similar range of viable growth values was obtained in other *Bacillus* species studied (Table 1). This shows that high values of viable bacterial cells were obtained in corn-starch in contrast to other substrate medium.

Soluble and corn starch are two major substrate considered for enzyme (amylase) production in this study. Table 4 shows the enzymatic activity of the amylolytic *Bacillus* species utilizing soluble starch as carbon sources, according to the basal medium described by Takasaki (1976). The result showed that most of the strains are able to produce considerable amount of amylase. *Bacillus subtilis* recorded 6.24 enzyme unit/ml broth and corresponding 68.0mg/ml maltose yield. *B. licheniformis* 4.2 unit/ml with 46.4 mg/ml maltose yield. *B. macerans* (S2) 3.0 unit/ml and 32.0mg/ml maltose was obtained as the enzymatic product. *Bacillus polymyxa, B.circulans* and *B. megaterium* shows low amylolytic activity as observed in Table 2.

Many *Bacillus* spp. Shows good enzymatic activity with the use of corn starch buffered substrate as substitute to determine its viability as a cheap carbon source. For instance, *B. macerans* (S2) recorded a value of 2.4 unit/ml while *B. licheniformis* (S2) and *B. subtilis* recorded a value of 2.0 unit/ml each. Other Bacillus Spp. Showed low enzymatic activity (Table 2).

Based on the research data cornstarch revealed the saccharifying ability of some *Bacillus* spp. (Table 1&2), Table 3 shows that *B. subtilis* produced the highest yield of amylases (6.24 unit/ml) that constitute both a and ß- amylases. The percentage of ß- amylase produced by *B. subtilis* was 92.35% *B. licheniformis* (S2) produced 100% (4.2 unit/ml) ß-amylase while *B. macerans* (S2) also produced 100% (3.0 unit/ml) of ß-amylase enzyme.

Table 1. Comparative utilization of carbon sources by amylolytic Bacillus spp. in three growth media

		Total bacterial count (cfu x 10^2 /ml)		
Source	Bacillus spp.	Corn starch substrate	Soluble starch substrate	Nutrient broth medium
Soil, U.I.	*B. macerans* (S1)	20.0	15.0	0.6
Canned milk Ibadan	*B. macerans* (S2)	25.0	20.0	9.0

Soil U.I	*B. licheniformis* (S1)	7.0	6.0	0.5
Wastewater U.I	*B. licheniformis* (S2)	20.0	5.0	18.0
Soil, U.I	*B. circulans* (S1)	2.0	1.8	2.0
Wastewater U.I	*B. circulans* (S2)	16.0	34.0	2.7
Canned milk Ibadan	*B. coagulans*	6.0	2.0	1.0
Waste water U.I	*B. polymyxa*	8.0	7.4	15.0
Waste water U.I	*B. subtilis*	9.0	7.0	19.0
ATCC (USA)	*B. cereus*	2.5	1.9	1.7
Mean		**11.58**	**10.1**	**7.4**

Table 2. Comparative yield of amylase by various strains of *Bacillus* in soluble starch and corn starch media

	Soluble starch		Corn starch	
Bacillus species	**Amylase (unit/ml)**	**Maltose (mg/ml)**	**Amylase (unit/ml)**	**Maltose (mg/ml)**
B. macerans (S1)	1.56	17.4	0.2	2.0
B. macerans (S2)	3.0	32.0	2.4	24.0
B.licheniformis (S1)	0.12	1.2	0.8	8.0
B. licheniformis (S2)	4.2	46.4	2.0	20.0
B. circulans	0.72	8.0	-	-
B. coagulans	0.84	9.6	-	-
B. subtilis	6.24	68.0	2.0	20.0

- Low enzyme activity

Table 3. Determination of Alpha and Beta - Amylases in Total Amylases produced by the Bacillus spp.

Bacillus species	**Total Amylase (Enzyme unit/ml)**	a **-Amylase (unit/ml)**	**ß -Amylase (unit/ml)**	**Percentage (%) ß - Amylase**
B. macerans (S1)	1.56	0.12	1.44	92.31
B. macerans (S2)	3.0	-	3.0	100.00
B. licheniformis (S1)	0.84	0.48	0.36	4.27
B. licheniformis (S2)	0.12	-	0.12	100.00
B. circulans (S1)	4.2	0.72	4.2	100.00
B. circulans (S2)	1.68	-	0.96	67.57
B. coagulans	0.72	-	0.72	100.00

B. subtilis	6.24	0.6	5.64	92.35

Table 4. Effect of two cations-Calcium Ca 2+ and Sodium (Na +) on amylase activity in soluble starch

		Ca^{2+}	Na^{+}
Bacillus species	**Amylase unit/ml**	**Amylase (unit/ml)**	**Amylase (unit/ml)**
B. macerans	3.0	1.32	2.28
B. licheniformis (S1)	4.2	1.88	N.D
B. licheniformis (S2 *)*	0.72	-	N.D
B. circulans (S1)	0.48	2.64	0.72
B. megaterium	-	0.12	-
B. polymyxa	-	0.72	-
B. subtilis	6.24	2.40	-

- *Low activity; ND = Not determine*

Other strains that produced mainly ß-amylase are as shown in Table 3. Metal ions of calcium ca2+ added to the assay system improve ß-amylase activity. Na+ was less effective for the enzymes activity (Table 4).

DISCUSSION

The scientific advancement of formulating substrates like corn-starch medium for ß amylase production from amylolytic *Bacillus* spp. was intensified in the result of this study. The results obtained showed the saccharifying power of various *Bacillus spp.* as a cheap source.

The Ten (10) amylolytic *Bacillus* species isolated from soil, waste-water and selected food sources belong to the genus *Bacillus,* this include two strains each of *Bacillus macerans* B *. licheniformis and B. circulans.* Others are *B. coagulans, B. megaterium, B. polymyxa and B. subtilis. B. cereus* ATCC 11778 was a type species. In a quantitative analyses to determine the viability of corn starch as a cheap carbon substrate for amylolytic *Bacillus* species. The microbes thrived well in corn-starch substrate compared with soluble starch and nutrient broth. The mean total bacterial count for 24 hours culture of all the bacillus isolates in corn starch substrate medium was 11.5 x 10 2 cfu/ml, soluble starch 10.1 x 10 2 cfu/ml. And nutrient broth medium 7.4 x 10 2 cfu/ml. This agreed with the work of Hensley et al. (1980) which reported that selected strains of Bacillus species, like *B. macerans* produce good yields of ß-amylase with corn steep languor. Srivastava and Baruah (1986) similarly reported that among various complex media tried for good amylase yield, corn steep liquor was found to be the best, but in the two cases they claimed that the corn liquor served as the principal nitrogen source. The disadvantages of the corn steep liquor were that it contains many chemical ingredients. It therefore became necessary to replace corn steep liquor with synthetic material of known chemical composition (Srivastava and Baruah, 1986).

Most of the *Bacillus* species studied had earlier been reported for ß-amylase synthesis or production. ß-amylase was found to be produced by some *Bacillus* spp. identified as *B. megaterium* (Hoshino et al. 1975 and Takasaki, 1976) and *B. circulans* (Hensley et al 1980). *B. macerans* was also reported for the production of enzymes having ß-amylase activity (Rose, 1980 and Priests, 1977).

In the *Bacillus* species among the active ß-amylase producers that show good enzymatic activity in the study were *B. licheniformis* (S2) 4.2 unit/ml, B. macerans (S2) 3.0 unit/ml and *B. circulans (S2)* 1.68 unit/ml. *B. macerans* (S2) 3.0 unit/ml and *B. circulans* (S1) 1.68 unit/ml

B. subtilis showed high enzymatic activity of 6.24 unit/ml but this constitute 5.64 unit/ml of ß-amylase. Low

value of ß-amylase activity was recorded in *B. megaterium* (0.12 unit/ml) and *B. coagulans* produced 0.36 unit/ml ß-amylase out of its total 0.84 unit/ml amylolytic activity (Table 3). The production of ß-amylase accompanied with some a-amylase in some *Bacillus spp.* is consistent with the findings of Hensley *et al* . (1980).

In conclusion, this study helps to formulate the use of corn-starch as a cheap carbon substrate medium for improving the production activities of ß-amylase from the genus *Bacillus.* The Ca^{2+}ion added during the assay systems generally improved ß-amylase production activity. Na+ was less effective for the enzyme activity.

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