African Journal of Biotechnology Vol. 5 (5), pp. 440-444, 1 March 2006
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2006 Academic Journals

*Full Length Research Paper*

# Growth pattern and structural nature of amylases produced by some *Bacillus species* in starchy substrates

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Accepted 6 January, 2006

**The growth pattern and microbial biomass formed during metabolic activities of the *Bacillus* species on starchy substrates was determined. The result showed that the strains *B. subtilis* (WBS), *B. licheniformis* (WBL) and *B. coagulans* (MBC) generally had high growth rate. *B. circulans* (SBC) and *B. coagulans* (WBC) has specific affinity for growth and some enzymatic activity on corn starch medium compared with other lower growth observed in *B. polymyxa* (WBP) but higher enzyme production. The amylolytic *Bacillus species* obtained utilized white corn starch substrate as a sole carbon source as well as soluble starch. The amylase production values range from 0.022 x $10^2$ unit/cfu in *B. circulans* (WBC) to 0.912 x $10^2$ unit/cfu in *B. licheniformis* (WBL) for corn starch, and 0.01 x $10^2$ unit/cfu in both *B. megaterium* (SBG) and *B. licheniformis* (SBL) to 0.693 x $10^2$ unit/cfu in *B. subtilis* (WBS) for soluble starch.**

**Key words:** Activity, *Bacillus*, enzymatic, metabolic, starchy, substrate.

## INTRODUCTION

*Bacillus* species are heterogeneous forms of organism and they are very versatile in their adaptability to the environment. There are various factors that influence the nature of their metabolic process and enzyme produced. Culture media components greatly affect the growth and fungal species. It was also reported that the medium composition affects amylase production as well as sporulation in some *Bacillus* species (Strivastava and Baruah, 1986).

Starch induces amylase production but there are reports indicating that starch may not be required for amylase production probably in organisms having constitutive enzymes (Tobey and Yosten, 1977; Strivastava and Baruah, 1986; Burbidge and Collier, 1958). Thus the nature of substrate including the nitrogen source and mineral element components of culture medium affects the metabolic process in microorganisms.

Optimization of cultural condition is important for maximum production of microbial strains (Bezbaruah et al., 1994). In this regard, appropriate media components and suitable conditions must be attained for optimal production of required products. In starch processing industries, immobilized cells are used to optimally exploit the amylase producing machinery of the cell, of which the β-amylase producing cells are employed for bioconversion of starch to maltose (Ray et al., 1995). Bacillus species and other forms of microorganisms grow at different rate with specificity to different substrates in culture medium. The growth conditions also influence their enzymatic activities (Prescott et al., 2002).

### MATERIALS AND METHODS

#### Microorganisms

The Bacillus strains for this study were obtained from wastewater, soil and milk sources in Ibadan Oyo State, Nigeria. A sporulating chemically defined medium was employed to aid the suitable growth and recovery of *Bacillus* species were as described by Leicth and Collier (1996). Amylolytic *Bacillus* species were selected for final study by using starch hydrolysis procedure (Cowan and

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Steel, 1985; Difco manual, 1984; Kotzekidou, 1996).

### Culture medium

Each organism was sub-cultured in nutrient agar medium and incubated for 24 h at 35ºC. Loopfuls of each sample was transferred into test tube containing sterile distilled water and thoroughly mixed and serially diluted to provide a homogeneous liquid suspension which served as inoculum containing an estimated $10^6$/cfu/ml of broth. Pour plate count technique and microscopy was used for the estimate as described by Giffel et al. (1995). Samples were plated out immediately.

A nutrient broth medium and other two nutrient broth preparations supplemented with corn starch and soluble starch carbon sources were used as the comparative cultures. Precisely, 10 ml each of culture media were inoculated with 1 ml of the inoculum and incubated at 37ºC for 24 h. The samples were initially serially diluted (10 fold) with sterile distilled water and 1 ml of appropriate sample was plated on plate count agar medium. A pour plate technique was employed in each instance to determine their growth pattern on soluble starch substrate and white corn starch substrates for appropriate assessment. Incubation was for 48 h.

The growth pattern of the *Bacillus* strains were studied by culturing the samples in different media supplemented with corn starch and soluble starch compared with the nutrient broth medium that served as a base medium. One ml of the appropriate dilution with similar range of count was inoculated into nutrient broth base medium supplemented with different carbon sources specified above and a nutrient broth base without supplement. This was cultured for 24 h at 37°C. 10 fold dilutions was done for each sample and analyzed at 6 to 24 h intervals using a spectrophotometer at 610 nm wavelengths.

### Crude enzyme preparation

Amylolytic bacterial isolates recovered from sampled sources were each cultured in a 50 ml broth medium containing (w/v) peptone (2%), ptarch (0.5%), $K_2HPO_4$ (0.3%) and $MgSO_4.7H_2O$ (0.1%) in Erlenmeyer flask of 200 ml capacity. The incubation was carried out for 40 h at 30ºC on a rotatory shaker (Model G24 Environmental incubator shaker, N.J., U.S.A.) at 150 rpm. The cultivated cells were removed by centrifugation for 15 min at 4000 rpm and resultant supernatant was used as the enzyme source.

The determination of the capability to release reducing sugar was by dinitrosalicylic acid (DNSA) method (Murao et al., 1979; Bailey, 1988) as described below in the next sub-section.

### Reducing sugar determination by DNSA method

The saccharifying ability of the crude enzyme was determined on 1.0% soluble starch and white corn starch substrate dissolved in phosphate buffer (pH 7.0). A measure of 0.1 milliliter of the crude enzyme was added to 1 ml of the substrate. After incubation for 10 min at 37ºC the reaction mixture was stopped by adding 2 ml of DNSA reagent (Murao et al., 1978). The reaction mixture was heated at 100ºC for 10 min and cooled. Then 17 ml of distilled water was added to the solution. The reaction mixture was allowed to stand for 15 min at room temperature. The optical density of each sample was measured using a spectrophotometer (Model Pye Unicam, U.S.A). The spectrophotometer was set up in a regulated environment usually with air conditioner and allowed to warm up for 15 min to enhance accurate reading. The optical meter gauge was standardized with a blank and control sample put into a cuvette that was fixed appropriately into the spectrophotometer. The control sample was buffered substrate solution compared with test enzyme sample to give corresponding values for estimation of reducing sugar released at 530 nm.

### Determination and separation of α- and β-amylase

The determination and separation of α- and β-amylase in crude enzyme produced by the *Bacillus* cultures was attained with the use of temperature and pH factors. The ratio of the β-amylase in the crude enzyme was first determined by removing the heat-labile β-amylase in little fraction of the enzyme assayed by subjecting the enzyme solution to heating in water bath thermostatically controlled at 70ºC for 15 min.

Since β-amylase is the enzyme of interest in the study, the pH favorable for the enzyme production was therefore found suitable for this purpose. Lower pH values of around 4.1 and pH 5.8 facilitates the production of α -amylase while upper pH values of pH 7 to pH 9 which favoured the production of β-amylase and elimination of α-amylase in the assay systems were considered. The enzyme was assayed using different pH in the substrate buffer medium.

## RESULT

*Bacillus* species that were catalase positive and have the ability to hydrolyse starch were finally selected for the study. The pattern of growth of the organisms on starchy substrate using spectrophotometer at wavelength 610 nm showed that the strains *B. subtilis* (WBS), *B. licheniformis* (WBL) and *B. coagulans* (MBC) have high growth rate. *B. circulans* (SBC) and *B. circulans* (WBC) has specific affinity for growth and enzymatic activity on corn starch. Most of the *Bacillus* strains had high growth values in corn starch substrate at 48 h and 54 h. This growth pattern was also observable in soluble starch substrate with lower growth indices. Few strains such as *B. polymyxa* (WBP) and *B. subtilis* (WBS) experienced some declination in corn starch medium at 24 h from 0.428 and 1.016 units to 0.422 and 0.775 units in the 54 h, respectively. The nutrient broth control sample generally show lower growth value compared with the starchy medium (Table 1).

The growth pattern and utilization of corn starch, as a carbon substrate by the amylolytic *Bacillus* species for the purpose of its use on large scale bases was further determined in the assay systems. Table 2 shows the comparative amylase production by soluble starch and cornstarch carbon sources. Ten of the *Bacillus* species studied are better on corn starch than soluble starch except in three strains which are *B. macerans* (MBM), *B. macerans* (SMB2) and *B. subtilis* (WBS). The amylase production values range from 0.022 x $10^2$ unit/cfu in *B. circulans* (WBC) to 0.912 x $10^2$ unit/cfu in *B. licheniformis* (WBL) for cornstarch and 0.01 x $10^2$ unit/cfu in both *B. megaterium* (SBG) and *B. licheniformis* (SBL) to 0.693 x $10^2$ unit/cfu in *B. subtilis* (WBS) for soluble starch (Table 2).

**Table 1.** Assessment of carbon sources utilization by the amylolytic *Bacillus* species.

| Sources | Strain code | *Bacillus* species | Total bacterial count (cfu x $10^2$/ml) | | |
|---|---|---|---|---|---|
| | | | Corn starch medium | Soluble starch medium | Nutrient broth base |
| Soil. U.I | SBM | *B. macerans* | 8.0 | 5.0 | 4.0 |
| Canned Milk, Ibadan | MBM | *B. macerans* | 25.0 | 20.0 | 9.0 |
| Soil, U.I | SBM1 | *B. macerans* | 16.0 | 15.0 | 0.6 |
| Soil, U.I | SBM2 | *B. macerans* | 17.4 | 1.5 | 5.0 |
| Wastewater, U.I | WBC | *B. coagulans* | 3.0 | 3.0 | 0.4 |
| Canned Milk, Ibadan | MBC | *B. coagulans* | 3.0 | 2.0 | 1.0 |
| Soil, U.I | SBL | *B. licheniformis* | 7.0 | 6.0 | 0.5 |
| Wastewater, U.I | WBL | *B. licheniformis* | 6.0 | 5.0 | 8.0 |
| Soil, U.I | SBC | *B. circulans* | 2.0 | 1.8 | 2.0 |
| Wastewater U.I | WBC1 | *B. circulans* | 16.0 | 34.0 | 2.7 |
| Soil, U.I | SBG | *B. megaterium* | 12.0 | 11.0 | 12.0 |
| Wastewater, U.I | WBP | *B. polymyxa* | 8.0 | 7.4 | 15.0 |
| Wastewater, U.I | WBS | *B. subtilis* | 9.0 | 7.0 | 19.0 |
| Type strain | ATCC 11778 | *B. cereus* | 2.5 | 1.9 | 1.7 |
| **Mean total bacterial count** | | | **8.49** | **7.54** | **5.77** |

**Table 2.** Comparative enzyme production in soluble starch and corn starch.

| Strain code | *Bacillus* species | Corn starch medium | | Soluble starch medium | |
|---|---|---|---|---|---|
| | | *Bacillus* population (x $10^2$ cfu) | Amylase (x $10^2$ unit/cfu) | *Bacillus* population (x $10^2$ cfu) | Amylase (x $10^2$ unit/cfu) |
| SBM | *B. macerans* | 8.0 | 0.165 | 5.0 | 0.144 |
| MBM | *B. macerans* | 25.0 | 0.072 | 20.0 | 0.15 |
| SBM1 | *B. macerans* | 16.0 | 0.112 | 15.0 | 0.104 |
| SBM2 | *B. macerans* | 17.4 | 0.41 | 1.5 | 1.2 |
| WBC | *B. coagulans* | 3.0 | 0.24 | 3.0 | 0.12 |
| MBC | *B. coagulans* | 3.0 | 0.44 | 2.0 | 0.42 |
| SBL | *B. licheniformis* | 7.0 | 0.102 | 6.0 | 0.02 |
| WBL | *B. licheniformis* | 6.0 | 0.912 | 5.0 | 0.84 |
| SBC | *B. circulans* | 2.0 | 0.36 | 1.8 | 0.06 |
| WBCI | *B. circulans* | 16.0 | 0.022 | 34.0 | 0.02 |
| SBG | *B. megaterium* | 12.0 | 0.06 | 11.0 | 0.01 |
| WBP | *B. polymyxa* | 8 | 0.06 | 7.4 | 0.016 |
| WBS | *B. subtilis* | 9.0 | 0.08 | 7.0 | 0.89 |

### Structural nature of amylases produced

All *Bacillus* strains studied produced β-amylase ranging from 0.12 unit/ml in *B. licheniformis* (SBL), *B. megaterium* (SBG) and *B. polymyxa* (WBP) to 5.64 unit/ml in *B. subtilis* (WBS) as shown in Table 3. α-Amylase was detected in some of the samples tested, ranging from 0.1 unit/ml in *B. macerans* (SBM1) to 1.30 unit/ml in *B. macerans* (SBM2). In general percentage (%) ratio of β-amylase was more than 60% in eleven out of the thirteen isolates studied. The only exception was in *B. macerans* (SBM2), 38.5% and *B. coagulans* (MBC) 42.7% β-amylase content (Table 3). The amount of α-amylase present in the amylase secreted by the microorganism was determined after heat treatment that denatured the heat-labile β-amylase. It was deduced from the result that *B. subtilis* with 0.6 unit/ml *B. circulans* (SBC) with 0.72 unit/ml and *B. macerans* (SBM2) with 1.30 unit/ml

**Table 3.** α-Amylase and β-amylase activities in the total amylase produced by the isolated *Bacillus* species.

| Strain code | *Bacillus species* | Total amylase (unit/ml) | α-Amylase (unit/ml) | β-Amylase (unit/ml) | Percentage (%) of β-amylase |
|---|---|---|---|---|---|
| SBM | *B. macerans* | 0.72 | * | 0.72 | 100 |
| MBM | *B. macerans* | 3.0 | * | 3.0 | 100 |
| SBM1 | *B. macerans* | 1.56 | 0.12 | 1.44 | 92.31 |
| SBM2 | *B. macerans* | 1.80 | 1.30 | 0.50 | 38.5 |
| WBC | *B. coagulans* | 0.36 | * | 0.36 | 100 |
| MBC | *B. coagulans* | 0.84 | 0.48 | 0.36 | 42.7 |
| SBL | *B. licheniformis* | 0.12 | * | 0.12 | 100 |
| WBL | *B. licheniformis* | 4.2 | * | 4.2 | 100 |
| SBC | *B. circulans* | 1.68 | 0.72 | 0.96 | 67.57 |
| WBC | *B. circulans* | 0.72 | * | 0.72 | 100 |
| SBG | *B. megaterium* | 0.12 | * | 0.12 | 100 |
| WBP | *B. polymyxa* | 0.12 | * | 0.12 | 100 |
| WBS | *B. subtilis* | 6.24 | 0.6 | 5.64 | 92.35 |

* Not detected.

showed high α-amylase activity. Other strains where some amount of α-amylase was detected were *B. macerans* (SBM1) with 1.2 unit/ml and *B. coagulans* (MBC) with 0.48 unit/ml. β-amylase from *Bacillus* species are usually accompanied with some α-amylase. Table 3 therefore shows the percentage (%) ratio of β-amylase produced compared with α-amylase content of the crude enzyme. *B. licheniformis* (WBL), *B. macerans* (MBM) and *B. circulans* (WBC) served as active β-amylase producers among tested strains because they have good yield of β-amylases without traces of α-amylase. The β-amylases of 0.48 unit/ml produced by *B. polymyxa* was low. Similarly, *B. coagulans* (WBC), *B. licheniformis* (SBL) and *B. megaterium* (SBG) each showed low enzyme recovery from the culture supernatant. *B. subtilis* produced the highest yield of 6.24 unit/ml amylases that have same traces of α-amylase with the β-amylase making 92.35% of the crude enzyme content.

## DISCUSSION

The Bacillus species actively utilized corn starch as a viable carbon source for metabolism. Similar growth pattern was observed in soluble starch but with lesser value, while the nutrient broths, which serve as control generally, recorded low growth range, compared with the starchy substrates.

The results above agreed with report of Hensley et al. (1980) who reported that selected strains of *Bacillus* species, like *B. circulans* could produce good yields of β-amylase with corn steep liquor. Srivastava and Baruah (1986) reported that among various complex media tried for good amylase yield, corn steep liquor was found to be the best. The disadvantage of the corn steep liquor was that it contains many chemical ingredients, and it was difficult to ascertain which of them induced amylase production. Therefore, the use of chemically defined medium as in this study is required for enzyme production studies (Srivastava and Baruah, 1986). Some amylolytic enzymes of *B. macerans* were active in starch-containing media, and the enzyme accumulated as the concentration of the carbon source declines (Priest, 1977). *B. macerans* was encountered among the amylolytic *Bacillus* species for this study.

Except for few *Bacillus* strains, most of the *Bacillus species* isolated in this study had earlier been reported for β-amylase production. β-amylase was found to be produced by *Bacillus polymyxa* during the year 1946 and there after and by a *Bacillus* spp. later identified as *B. megaterium* (Hoshino et al., 1975; Takasaki, 1976a) and *B. circulans* (Hensley et al., 1980). *B. macerans* was also reported to produce enzymes having β-amylase activity (Rose, 1980 and Priest, 1977).

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