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Growth Measurement of Some Amylolytic *Bacillus* Species in Three Media

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Abstract. Study of the growth pattern of some *Bacillus* species on starchy substrates showed that the metabolic activity affected the enzymatic activity. *B. subtilis* (WBS), *B. licheniformis* (WBL) and *B. coagulans* (MBC) generally had higher growth rate. *B. circulans* (SBC) and *B. coagulans* (WBC) had higher growth on cornstarch medium with corresponding higher ɓ-amylase production as compared to other strains such as *B. polymyxa.* Ten of the 13 *Bacillus* species studied had better performance on cornstarch than on soluble starch except *B. macerans* (MBM), *B. macerans* (SMB2) and *B. subtilis* (WBS). The enzyme production ranged from 0.022 unit/cfu x 10^2 to 0.912 unit/cfu x 10^2 on cornstarch and 0.01 unit/cfu x 10^2 to 0.693 unit/cfu x 10^2 on soluble starch. Relatively higher â-amylase activity was observed in *B. subtilis, B. licheniformis, B. macerans* and *B. circulans* (WBC1).

Keywords: *Bacillus* sp., starch, beta amylase production, enzymatic activity

Introduction

Many environmental factors and culture media components greatly affect the metabolic processes in microorganisms. Ajayi and Fagade (2006), Lin *et al.* (1997) and Amoa-Awua and Jakobsen (1995), in their study demonstrated the metabolic activity of some microbial strains and the corresponding enzymatic productivity. Previous researches have also shown that medium composition affects enzymatic activities as well as sporulation in some microorganisms including *Bacillus* sp., (Ajayi and Fagade, 2006; Ray *et al.*, 1995). Starch induces amylase production but there are reports indicating that starch may not be required for amylase production probably in organisms having constitutive enzymes (Shittu *et el.*, 2005; Srivastava and Baruah, 1986; Burbidge and Collier, 1958). Thus the nature of substrate, including the nitrogen source and mineral element components of culture medium, affects metabolic processes in the microorganisms.

Bacillus species and other forms of microorganisms grow at different rates with specificity to different substrates in culture medium (Tobey and Yosten, 1977). The growth conditions also influence their enzymatic activities (Nortermann, 1992). Generally, media composition, cultural conditions, microbial cell biochemistry and physiology play vital roles in amylase producing mechanisms of *Bacillus* species (Bezbaruah *et al.*, 1994; 1987).

In the present work, study was made of the growth of 13 amylase producing *Bacillus* species on starch and their corresponding amylase production activity, also with reference to carbon source.

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Materials and Methods

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, as described by Leicth and Collier (1996). Amylolytic *Bacillus* sp., were identified by standard microbiological techniques (Kotzekidou, 1996) and selected for final study by using starch hydrolysis procedure (Cowan and Steel, 1985; Difco, 1984).

Each organism was sub-cultured in nutrient agar medium and incubated for 24 h at 35 °C. Loopful of each sample was transferred to test tube containing sterile distilled water, thoroughly mixed and serially diluted to provide a homogeneous liquid suspension to be used as inoculum containing an estimated 10^6/cfu/ml of broth. Pour plate count technique and microscopy was used for the estimate. Samples were plated out immediately.

The growth pattern of *Bacillus* strains were studied by culturing the samples in different media supplemented with cornstarch, soluble starch and compared with the nutrient broth medium that served as the 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 the nutrient broth base without supplement. This was cultured for 24 h at 37 °C. Ten fold dilution was made for each sample and analyzed at 6

to 24 h intervals using a spectrophotometer at 610 nm wavelength.

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

Determination of the saccharifying capability of the enzyme to release the reducing sugar was made by dinitrosalicylic acid (DNSA) method (Bailey, 1988; Murao *et al.*, 1979) as described below.

Soluble starch and white cornstarch substrate 1.0% were dissolved in phosphate buffer (pH 7.0). A measure of 0.1 ml of the crude enzyme was added to 1 ml of the substrate. After incubation for 10 min at 37 °C, the reaction mixture was stopped by adding 2 ml of DNSA reagent. The reaction mixture was heated at 100 °C for 10 min, cooled and then 17 ml of distilled water was added. The reaction mixture was allowed to stand for 15 min at the room temperature. Optical density of each sample was measured using a spectrophotometer (Model Pye Unicam, USA). 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 which was compared with the test enzyme sample to give corresponding values for estimation of reducing sugar released at 530 nm.

Results and Discussion

Bacillus species obtained from various sample sources such as soil, wastewater and food (milk) sources demonstrated relatively higher growth value on the cornstarch, compared with that on the soluble starch while the nutrient broth, which served as control, recorded low growth range as shown in Fig. 1 and Table 1. Ten *Bacillus* strains utilized cornstarch better than soluble starch for enzyme production except three namely *Bacillus macerans* (SBM1), *B. macerans* (MBM) and *B. subtilis* (WBS) (Table 2). The strains *B. subtilis* (WBS), *B. licheniformis* (WBL) and *B. coagulans* (MBC) generally had high growth rate. *B. circulans* (SBC) and *B. coagulans* (WBC) had specific affinity for growth and some enzymatic activity was observed on the cornstarch medium with high growth value of 1.118 and 1.080 units at 48 h. Correspondingly

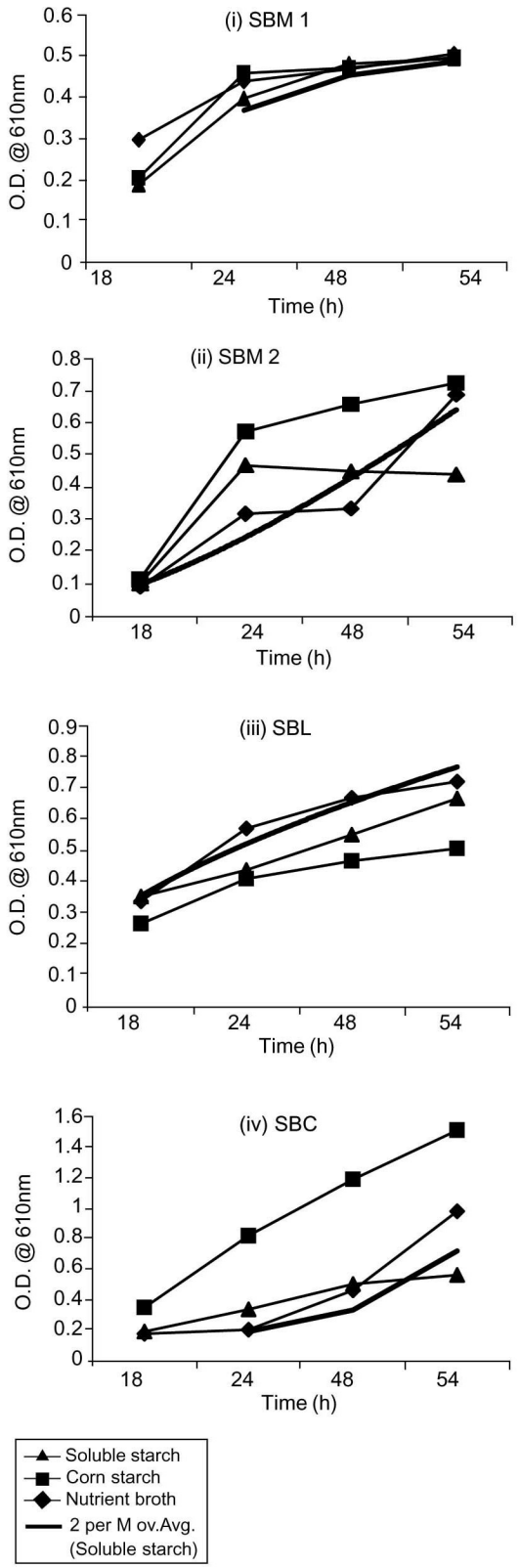

Fig. 1a(i-iv). Comparative (O.D. measurement)growth pattern of the isolated *Bacillus* species in different media. (Soil source: University of Ibadan, Nigeria).

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

Sources	Strain code	*Bacillus* species	Total bacterial count (CFU/ml x10^2)			SE (%)
			Corn starch substrate	Soluble starch substrate	Nutrient broth medium	
Soil, U.I.	SBM	*B. macerans*	8.0	5.0	4.0	12.25
Canned milk, Ibadan	MBM	*B. macerans*	25.0	20.0	9.0	15.16
Wastewater, U.I.	WBC	*B. coagulans*	3.0	3.0	0.4	22.50
Canned milk	MBC	*B. coagulans*	6.0	2.0	1.0	29.40
Soil, U.I.	SBL	*B. licheniformis*	7.0	6.0	0.5	27.80
Wastewater, U.I.	WBL	*B. licheniformis*	20.0	5.0	18.0	18.94
Soil, U.I.	SBC	*B. circulans*	2.0	1.8	2.0	1.98
Wastewater, U.I.	WBC	*B. circulans*	16.0	34.0	2.7	29.79
Soil, U.I.	SBG	*B. megaterium*	12.0	11.0	12.0	1.66
Wastewater, U.I.	WBP	*B. polymyxa*	8.0	7.4	15.0	13.90
Wastewater, U.I.	WBS	*B. subtilis*	9.0	7.0	19.0	18.37
ATCC, (USA)	ATCC 11778	*B. cereus*	2.5	1.9	1.7	6.32
	Mean		9.87	8.67	7.11	10.73

SE = standard error; U.I. = University of Ibadan, Nigeria

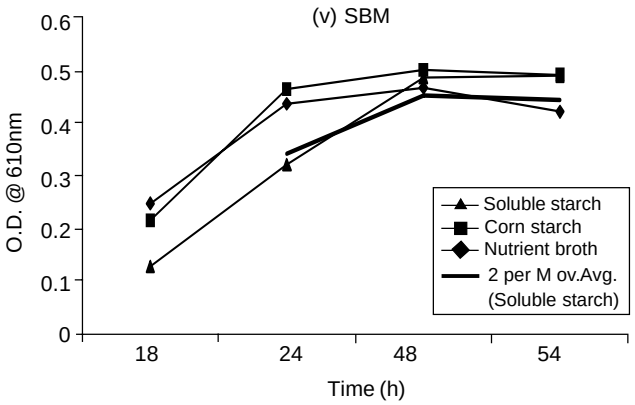

Fig. 1a(v). Comparative (O.D. measurement)growth pattern of the isolated *Bacillus*s pecies in different media. (Soil Source: University of Ibadan, Nigeria)

ь-amylase production was higher with values of 0.606 unit/O.D. and 0.667 unit/O.D., respectively, as compared to the other lower growth indices observed in *B. polymyxa* (WBP) of 0.425 unit but higher enzyme production value of 1.129 unit/O.D (Fig. 1).

The *Bacillus* species demonstrated different patterns of growth rates with relatively higher values in starchy medium (Fig. 1a-c; Table 1). The enzyme production values ranged from 0.022 unit/cfu x 10^2 by *B. circulans* (WBC) to 0.912 unit/cfu x 10^2 by *B. licheniformis* (WBL) for cornstarch and 0.01 unit/cfu x 10^2 by both *B. megaterium* (SBG) and *B. licheniformis* (SBL) to 0.693 unit/cfu x 10^2 by *B. subtilis* (WBS) for soluble starch (Table 2). These results agree with those of Hensley *et al.* (1980) who reported good yields of ь-amylase on corn steep liquor among various complex media by selected strains of *Bacillus* species, like *B. circulans.* Srivastava and Baruah (1986) also found corn steep liquor 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 used in this study is required for enzyme production activities (Lederberg, 1992; 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). During the study, *B. macerans* was encountered among the amylolytic *Bacillus* species. In this study the organisms used have capabilities to produce amylase and this was influenced by the effect of the regulated conditions especially in the utilization of cornstarch substrates compared with other carbon sources. This greatly affected the quality or characteristics of the enzymes produced and it conformed with the studies of Montgomery *et al*. (1990) and Srivastava and Baruah (1986); they stated that the nature and characteristics of enzymes produced by different species of bacteria, depends on the strains of bacteria involved, moreover an optimal growth condition may be determined for each strain.

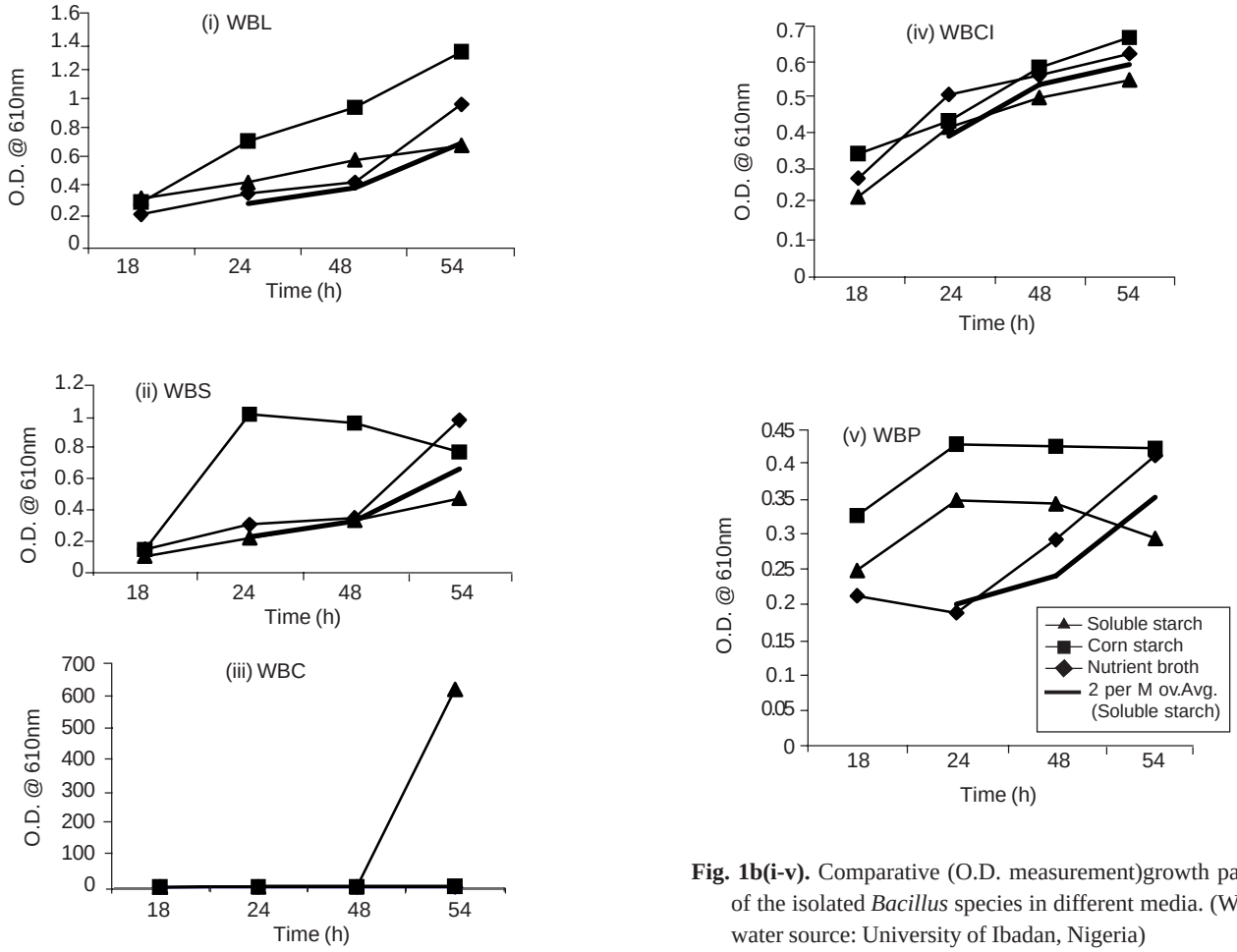

Fig. 1b(i-v). Comparative (O.D. measurement)growth pattern of the isolated *Bacillus* species in different media. (Waste-water source: University of Ibadan, Nigeria)

Table 2. Comparative enzyme production in soluble starch and cornstarch carbon sources

		Corn starch medium			Soluble starch medium		
Strain code	*Bacillus* species	Amylase (unit/ml)	*Bacillus* population (cfu x 10^2)	Amylase (unit/cfu x 10^2)	Amylase (unit/ml)	*Bacillus* population (cfu x 10^2)	Amylase (unit/cfu x 10^2)
SBM	*B. macerans*	1.32	8.0	0.165	0.72	5.0	0.144
MBM	*B. macerans*	1.80	25.0	0.072	3.0	20.0	0.15
SBM1	*B. macerans*	1.80	16.0	0.112	1.56	15.0	0.104
SBM2	*B. macerans*	0.72	17.4	0.41	1.80	1.5	1.2
WBC	*B. coagulans*	0.72	3.0	0.24	0.36	3.0	0.12
MBC	*B. coagulans*	1.32	3.0	0.44	0.84	2.0	0.42
SBL	*B. licheniformis*	0.72	7.0	0.102	0.12	6.0	0.02
WBL	*B. licheniformis*	4.56	6.0	0.912	4.2	5.0	0.84
SBC	*B. circulans*	0.72	2.0	0.36	0.12	1.8	0.06
WBCI	*B. circulans*	0.36	16.0	0.022	0.72	34.0	0.02
SBG	*B. megaterium*	0.72	12.0	0.06	0.12	11.0	0.01
WBP	*B. polymyxa*	0.48	8	0.06	0.12	7.4	0.016
WBS	*B. subtilis*	0.72	9.0	0.08	6.24	7.0	0.89

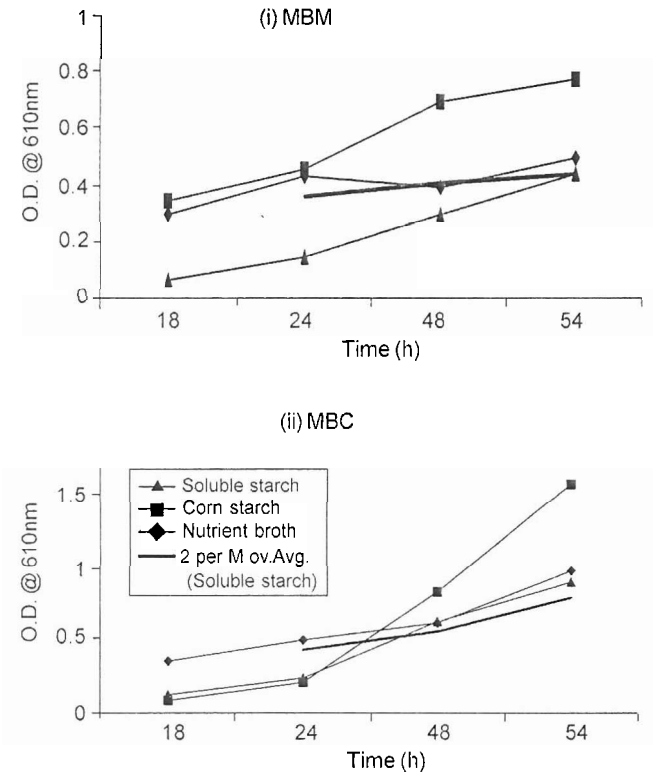

Fig. 1c. Comparative (O.D. measurement) growth pattern of the isolated *Bacillus* species in different media (canned milk).

The carbon sources used in the study were soluble starch, cornstarch, glucose and sucrose. They all influenced the activity of amylases (Table 3). Soluble starch carbon source

Table 3. Effect of carbon sources on amylase production (unit/ml) on soluble starch buffered substrate

Carbon substrate	SBM	MBM	MBC	WBL	WBS
Soluble starch	0.72	3.0	0.84	4.2	6.24
Corn starch	0.84	0.84	1.32	3.72	0.12
Glucose	0.84	0.84	0.6	0.72	1.20
Sucrose	0.24	0.72	–	0.48	0.96

favoured high enzymatic activity which ranged between 0.12 unit/ml for *B. licheniformis* (SBL strain), *B. megaterium* (SBG) and *B. polymyxa* (WBP) to 6.24 unit/ml by *B. subtilis* (WBS). Cornstarch substrate in the culture medium also recorded high yield of amylase ranging from 0.12 unit/ml for *B. macerans* (SBM2), *B. coagulans* (WBC), *B. licheniformis* (SBL), *B. megaterium* (SBG), *B. subtilis* (WBS) to 3.72 unit/ml by *B. licheniformis* (WBL). The enzymatic activity of the *Bacillus* strains with use of sucrose as carbon source was very low. This ranged from 0.24 unit/ml to 0.72 unit/ml among the three strains that showed some activity. Nevertheless, results with reference to the cornstarch buffered substrate varied (Fig. 1a-c).

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