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RESEARCH ARTICLE

SCREENING AND PRODUCTION OF MANNANASE BY *BACILLUS* STRAINS ISOLATED FROM FERMENTED FOOD CONDIMENTS

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Abstract

Bacillus strains isolated from 'Iru' and 'Ugba' was screened for mannanase production and production condition was optimized. Twenty two *Bacillus* strains isolated were *Bacillus subtilis* IE, *Bacillus pumilus* S5, *Bacillus licheniformis* S4, *Bacillus cereus* U9, *Bacillus megaterium* U1 and *Bacillus coagulans* U3. *Bacillus subtilis* IE had the highest frequency of occurrence (31.82%). After screening six strains were selected for mannanase production under optimized condition. pH 5.0 was optimum for mannanase production by *B. subtilis* IE (0.1600 U/ml) and *B. pumilus* S5 (0.1989 U/ml). Optimum growth was at pH 3 and 40^{o}C for all the isolates except *B. subtilis* IE. *B. subtilis* IE (0.0700U/ml) and *B. megaterium* UI (1.561) had the highest production and growth. 30 and 36 hours incubation time was optimum for mannanase production and growth. *B. subtilis* IE (0.203a U/ml) and *B. megaterium* UI (0.800 a) had the highest. Agitation condition, fructose and glucose induced optimum mannanase production and growth, it ranged from 0.042 – 0.1179 U/ml and 0.074 – 0.611 in which *B. subtilis* IE and *B. licheniformis* S4 had the highest. Urea and yeast extract was the best nitrogen source for mannanase production and growth by *B. subtilis* IE (0.198 U/ml) and *B. megaterium* UI (1.561).

Keywords: Mannanase, Fermented food condiments, *Bacillus* strains, optimized condition

Introduction

The Mannan endo-1,4-β-mannosidase or 1,4-β-D-mannanase (EC 3.2.1.78), commonly named β-mannanase, is an enzyme responsible for the transformation of heteromannans to manno-oligosaccharides and small amount of mannose, glucose and galactose. Mannanases are involved in catalyzing β-1, 4-mannosidic linkages in the main chain of β-1,4-mannans, glucomannans and galactomannans (Gubitz *et al.*, 2001, Sanchez, 2009). Mannanases are produced by variety of organisms such as bacteria, fungi, higher plants and animals (Dekker and Richard, 1976). Mannanases has various functions and industrial application including food technology; bioconversion of biomass wastes to fermentable sugars, upgrading of animal feed stuff to increase the digestibility, bio-bleaching of pulp, print processing for textiles, detergent industry and processing of coffee beans, marine algae and other plant materials (Chandrakant and Bisaria, 1998,

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Ray *et al*., 1982; Gubitz *et al*., 1997; Hashimoto and Fukumoto, 1969 and Clark *et al*., 1990).

Mannan is one of the major polysaccharides present in seeds of leguminous plant and plant cell walls, such as locust bean gum (LBG), copra meal, tuber of konjac, coffee beans and palm seeds.

Food condiments in Nigeria and many other countries of west and central Africa are popular strong smelling fermented food culinary products that give pleasant aroma to soups, sauces and other prepared dishes. They also have great potential as key protein, fatty acid and good sources of gross energy (Umoh and Oke, 1974). Therefore, condiments are basic ingredients for food supplementation and their socioeconomic importance cannot be over emphasized in many countries especially in Africa and India where protein calorie malnutrition is a major problem.

Some of the most important food condiments consumed in Nigeria include "Ogiri" which is produced from melon seeds Citrilus vulgaris; "iru" or "dawadawa" or "dadawa", produced from African locust bean Parkia biglobosa (Odunfa, 1985), "ugba", produced from oil bean seeds (Penthacletra macrophyla) (Obeta, 1985, Oyeyiola, 1981); "Ogiri-igbo", produced from castor oil seeds (Ricimus communis (Odunfa, 1986), "dadawa", from soya-beans (Ogbadu and Okagbue, 1988), "owoh" from cotton seeds (Gossypium hirsitum), and "Ogiri" made from (Parkia filicoidea). Most of the fermented vegetable proteins reported are from leguminous seeds, and of the thousands known legumes, less than twenty are used extensively today. Other leguminous seeds in common use include peanuts, soya beans, locust beans, oil beans, cowpeas, lentils, alfalfa (luceme). *Bacillus* strains have been involved in fermentation processes most especially food condiments. They are able to produce high potential proteases and mannanases, and their main activity during fermentation is enzymatic proteolysis and mannanolysis or hydrolysis of galactomannan present as a storage polymer in the seed of leguminous plants (Lee *et al*., 2005, Ouoba *et al*., 2004). Most *Bacillus* strains are not harmful to humans (Celandroni *et al*., 2000). *Bacillus* mannanase can be proving efficient in breaking down mannan into smaller units. Moreover, Bacillus fermentation can provide products with high nutrient availability (Odunfa, 1985).

Due to the scarcity of information on decomposition of mannan in leguminous plants by Bacillus species isolated from fermented food condiments, this project aims at isolation, characterization and production of mannanase by *Bacillus* strains isolated from fermented leguminous food condiments from different locations in Nigeria.

Materials and Methods

Sample collection

Traditionally fermented African oil bean ("ugba"), and "iru" were purchased from retailers at Bodija market in Ibadan Metropolis. All were transported to the laboratory in sterile polythene bags and stored at 4°C.

Isolation of Bacillus strains

One gram of the samples was mashed and homogenized. 1 ml of the homogenate was added to 9 ml sterile physiological saline and serially diluted. 1 ml of the diluents was plated in molten sterile nutrient agar (for total viable counts) and Tryptone soy agar (for spore formers) and the plates were incubated at 30^{o}C for 48 hours.

Representative colonies of the spore forming bacteria obtained within each plate after incubation were picked; sub-cultured with repeated streaking on nutrient agar and incubated for 24 hours at 30^{o}C to obtain pure cultures.

Screening of the isolates for mannanase production

The isolates were screened for mannanase producing ability by inoculating them in a sterile medium containing 1% Locus Bean Gum (LBG), 0.1% yeast extract, 0.1% peptone, 0.1% NH_4NO_3, 0.14% KH_2PO_4, 0.02% $MgCl_2$, 1% Congo Red and 3% Agar (Rattanasuk and Ketudat-Cairns, 2009). The plates were incubated at 30^{0}C for 24 hours, 48 hours and 72 hours. The mannanase activity of each isolate was measured based on the ratio of the diameter of the clearing zone formed to the

diameter of the colony. The colonies with highest clear zone were collected and maintained as frozen stocks in the presence of 20% glycerol at – 87°C.

Mannanase production in submerge fermentation

The Mannanase Production Medium (MPM) containing in g/l: Bacteriological peptone 0.1, yeast extract 0.1, $MgCl_2$ 0.02, KH_2PO_4 0.14, NH_4NO_3 0.1, and locust bean gum (LBG) 1.0, Distilled water 1 liter and pH 7.0 was used. 100 ml of the sterile mannanase production medium was inoculated with 0.5 ml of the isolate and incubated for 24 hrs. After incubation, the fermentation medium was harvested by centrifugation at 4000 rpm for 30 minutes at 4°C. The supernatant was used to assay for mannanase activity.

Mannanase assay and protein concentration

The mannanase activity was assayed by measuring the reducing sugars using dinitrosalicylic acid (DNS) method (Miller, 1959). An assay mixture containing 0.5 ml of the culture supernatant and 0.5 ml of 0.5% (w/v) LBG in 0.02M phosphate buffer at pH 7.0 was incubated at 50°C for 30 min. After incubation, 1 ml of DNS reagent was added and boiled for 5 – 15 min.

The developed red brown color was measured at 575 nm. The amount of reducing sugar released was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme producing 1 µ mole of mannose per minute under experimental conditions. Protein concentration was determined by the method of Lowry *et al*. (1951) with bovine serum albumin as a standard.

Mannanase production under optimized condition

The mannanase production under optimized condition by Bacillus strains was investigated. Mannanase production under different pH (3 – 11), temperature (25°C - 45°C), incubation time (12, 18, 24, 30, and 36 hours), static and agitation conditions, carbon (glucose maltose, sucrose, mannose and fructose) and nitrogen sources (urea, yeast, casein, NH_4NO_3, and NH_4SO_4) was investigated. Growth was determined by taking the optical density (OD), and the fermentation medium was analyzed for mannanase production.

Results and Discussion

Isolation and screening of the isolates

A total of twenty two *Bacillus* strains obtained from "ugba" and "iru" were identified as *B. subtilis* IE, *B. pumilus* S5, *B. licheniformis* S4, *B. cereus* U9, *B. megaterium* UI and *B. coagulans* U3. Identification was done using Bergy's Manual of Systematic Bacteriology (Sneath, 1986). The *Bacillus* strains and their frequency of occurrence are shown in Table 1. *B. suibtilis* IE had the highest frequency of occurrence (31.82%) followed by *B. pumilus* S5 (22.27%), while *B. cereus* had the least (4.55%) (Table1). The preliminary screening of the isolates for mannanase production on solid agar is shown in Table 2. All the tested *Bacillus* strains were able to hydrolyze locust bean gum during growth on agar plates, zones of different sizes were cleared around the strains. The highest mannanase activity (34 mm) was reached by *B. licheniformis* S4, followed by *B. suibtilis* IE and *B. pumilus* S5 (16 mm), *B. coagulans* U3 (15 mm), *B. megaterium* U1 (12 mm), B. cereus U9 had the least diameter of 10 mm after 72 hrs of incubation. In this study a total of 22 *Bacillus* isolated from "Iru" and "Ugba" has ability to degrade Locust Bean Gum and six strains were selected for further studies. *Bacillus* strains have long been used for the production of various fermented food condiments and industrial enzymes (Schallmey *et al*., 2004). Many strains of Bacillus are non pathogenic and have been referred to as GRAS (generally recognize as safe) status (Olempska-Beer *et al*., 2006). All the strains isolated from the fermented food condiments were *Bacillus* and they all have the ability to degrade LBG and were able to secret mannanase.

Table 1. *Frequency of occurrence (%) of the isolated Bacillus strains*

Isolate	Frequency of occurrence (%)
B. suibtilis IE	31.82
B. pumilus S5	27.27
B. lichenformis S4	22.72
B. coagulans U3	9.09
B. cereus U9	4.55
B. megaterium UI	4.55

Table 2. *Screening of the Bacillus strains for mannanase activity on solid agar plate (mm)*

Isolate name	Diameter of zone clearance (mm)		
	24 hours	48 hours	72 hours
B. cereus U9a	10	10	10
B. licheniformis S4a	14	14	14
B. coagulans U3a	10	20	20
B. subtilis IEa	15	15	15
B. licheniformis S4b	20	20	20
B. licheniformis S4c	19	19	19
B. megaterium UIa	12	12	12
B.pumilus S5a	13	13	13
B.pumilus S5b	12	12	12
B. subtilis IEb	15	15	15
B. licheniformis S4d	34	34	34
B. pumilus S5c	16	16	16
B. subtilis IEc	16	16	16
B. coagulans U3b	15	15	15
B.licheniformis S4e	30	30	30
B.pumilus S5d	11	11	11

The best six mannanase producing strains of Bacillus were selected for further studies under optimized condition. Mannan is a useful substrate in many industries and is the major polysaccharides of legume seeds, coconut kernel and tubers of konjac. Several mannanase producing bacteria and fungi have been reported (Dekker and Richard, 1976).

Mannanase production under optimized condition

Effect of pH on growth and enzyme production by the strains is shown in Figure 1a and 1b respectively. pH has a profound effect on growth of the *Bacillus* strains, growth ranged from 0.091f - 1.082a in which the optimum was recorded at pH 3.0 by *B. cereus* U9, *B. pumilus* S5 and *B. coagulans* U3, pH 9 by *B. licheniformis* S4 and pH 11.0 by *B. subtilis* IE.

There was a significant difference ($P \geq 0.05$) in the production of mannanase at different pH, production ranged from 0.016f - 0.1989a U/ml in which B. pumilus S5 had the optimum production at pH 5.0 followed by *B. subtilis* IE. Optimum production was also recorded at pH 9.0 by *B. licheniformis* S4.

Table 3 shows the total protein (mg/ml) produced by the *Bacillus* strains at different pH. It ranged from 0.120 - 0.242 mg/ml in which *B. megaterium* UI had the highest at pH 11.0. pH 5.0 was the optimal pH for mannanase production by *B. subtilis* IE and *B. pumilus* S5.

At pH 5.0 optimal mannanase production by *B. subtilis* IE and *B. pumilus* S5 was recorded. This is in agreement with the report of Mendoza *et al.* (1994) who reported that pH 5.0 was optimum for *B. subtilis* NM-3. This report is not in agreement with Jiang *et al.* (2006) who reported pH 6.0 as optimum for *B. subtilis*, Meenakshi *et al.* (2010) reported pH 6.5 for *Bacillus* sp. MG-33 and Zakaria *et al.* (1998) reported pH 7.0 for *B. subtilis* KU-1.

Effect of incubation temperature on optimum growth and mannanase production by *Bacillus* strains is shown in Figure 2a and 2b. Incubation temperature had a profound effect on growths of the isolates. The growth ranged from 0.020f - 1.561a in which the optimum was reached at 40^{o}C by *B. megaterium* UI followed by *B. cereus* U9. Incubation at 25^{o}C did not support the growth of *Bacillus* strains.

Manannase production ranged from 0.0093f - 0.0700a U/ml in which *B. subtilis* IE had the optimum production at 40oC followed in order by *B. pumilus* S5, *B. megaterium* UI, *B. coagulans* U3 and *B. cereus*. The least enzyme production was recorded by *B. licheniformis* S4 at 30^{o}C. Table 4 shows total protein (mg/ml) produced by the *Bacillus* strains at different incubation temperature, it ranged from 0.050 - 0.280 mg/ml.

The isolates grew optimally at 40^{o}C, this is good enough for enzyme production, but when temperature exceeds 40^{o}C there was drastic reduction in growth.

Reduction in growth at higher temperature may be as result of inhibition due to the dryness of the substrate and the fact that high temperature brought a negative impact towards metabolic activity of the organism.

Inability of the isolate to grow optimally at low temperature may be due to problem in spore proliferation as reported by Tunga *et al.* (1998).

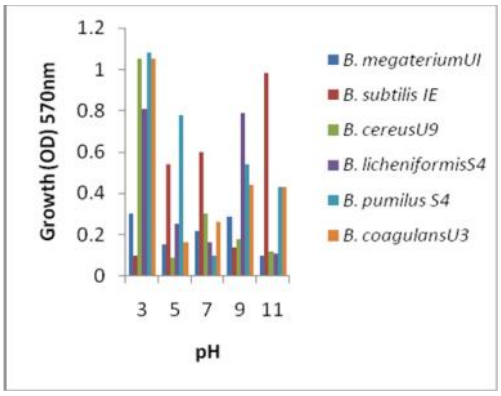

Figure1a. *Effect of pH on the growth of B. strains*

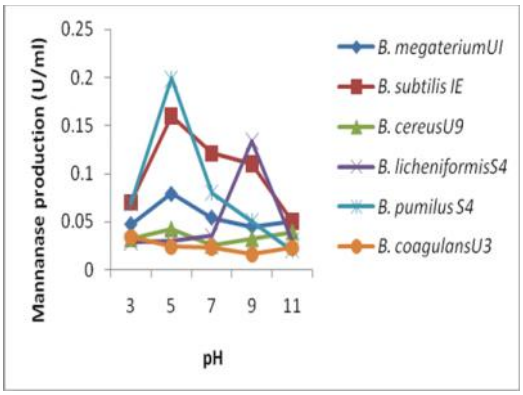

Figure1b. *Effect of pH on mannanase production by the B. strains*

Table 3. *Total Protein (mg/ml) produced by the Bacillus strains at different pH*

Isolate	pH				
	3	**5**	**7**	**9**	**11**
B. megaterium UI	0.150d	0.187b	0.192b	0.220a	0.242a
B. subtilis IE	0.180c	0.176c	0.182c	0.200b	0.202b
B. cereus U9	0.221a	0.187b	0.192b	0.190c	0.192c
B. licheniformis S4	0.190b	0.152d	0.162d	0.160d	0.163d
B. pumilus S5	0.180c	0.192a	0.220a	0.120f	0.127f
B. coagulans U3	0.180c	0.192a	0.220a	0.130e	0.130e

Data along the same column with different superscript alphabets are significantly different (P≥0.05)

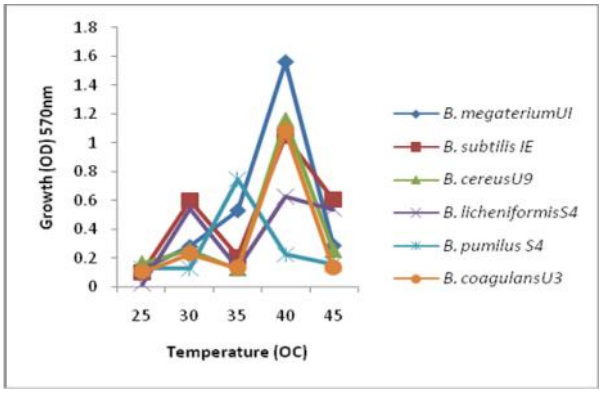

Figure2a. *Effect of temperature on the growth of B. strains*

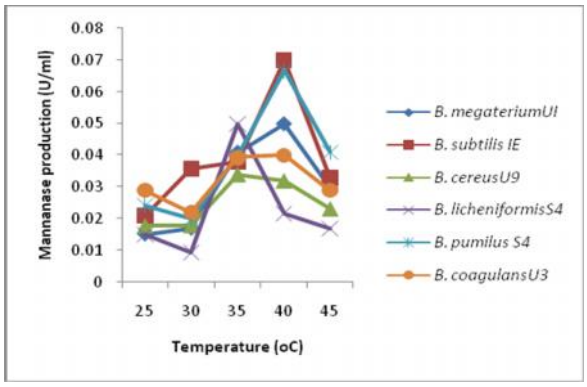

Figure2b. *Effect of temperature on mannanase production by B. strains*

Table 4. *Total Protein (mg/ml) produced by the Bacillus strains at different temperature (°C)*

Isolate	Temperature (° C)				
	25	**30**	**35**	**40**	**45**
B. megaterium UI	0.120e	0.100e	0.150e	0.170d	0.180b
B. suibtilis IE	0.181c	0.050f	0.270a	0.180c	0.160c
B. cereus U9	0.210b	0.280a	0.180d	0.150e	0.080e
B. licheniformis S4	0.140d	0.230b	0.100f	0.240b	0.160c
B. pumilus S5	0.100f	0.220c	0.210b	0.280a	0.110d
B. coagulans U3	0.230a	0.190d	0.200c	0.130f	0.230a

Data along the same column with different superscript alphabets are significantly different (P≥0.05)

In the temperature range of 30 – 40°C mannanase productions by *B. subtilis* IE and *B. pumilus* were optimum. This is in agreement with the report of Rattanasuk and Ketut-Cairns (2009) and Cao and Hu (2011) who reported that 30°C optimum for *Chryseobacterium indolgenes* and 30-40°C for Yeast. Higher temperature (50–60°C) was reported for *B. subtilis* strains NM-39, *B. subtilis* strains KU-1, *B. suibtilis* strains 5H and *Bacillus* sp. MG-33 (Mendoza *et al.*, 1994; Zakaria *et al.*, 1998 and Khanongnuch *et al.*, 1998, Meenakshi *et al.*, 2010; Zhang *et al.*, 2000).

Figure 3a and 3b shows the effect of incubation time on optimum growth and production of mannanse by *Bacillus* strains respectively. The growth ranged from 0.116f - 0.800a in which the optimum growth was recorded at 36 hours of incubation for all the isolates. *B. megaterium* UI had the optimum growth at 24 hours of incubation while 18, 24 and 30 hours of incubation did not favour growth of *B. subtilis* IE.

There was variation in mannanase production at different hours of incubation; it ranged from 0.0353f - 0.2033a U/ml in which the optimum production and the least was at 30 hours of incubation by *B. subtilis* IE and *B. coagulans* U3 respectively.

Effect of agitation and static condition on optimum growth and mannanase production is shown in Figure 4a and 4b respectively. The growth ranged from 0.136f - 1.825a in which *B. megaterium* UI had the optimum growth during agitation. Under agitation, mannanase production ranged from 0.0092f - 0.1390a U/ml in which *B. coagulans* U3 had the optimum production.

Agitation induced optimum mannanase production by *B. coagulans* U3 but did not support enzyme production by other *Bacillus strains*. Mannanase production under static condition ranged from 0.0354f - 0.2033a U/ml in which *B. subtilis* IE had the optimum during static condition. Static condition induced optimum yield of mannanase by *B. subtilis* IE followed by *B. licheniformis* S4.

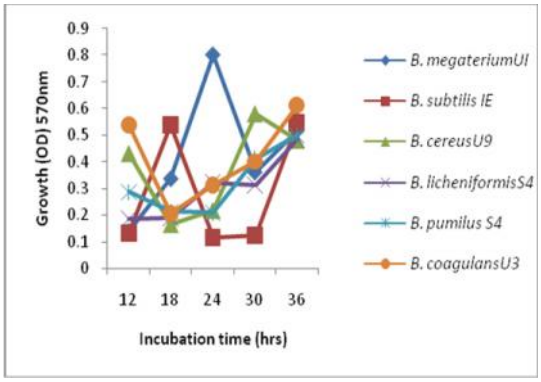

Figure 3a. *Effect of incubation time on the growth of B. strains*

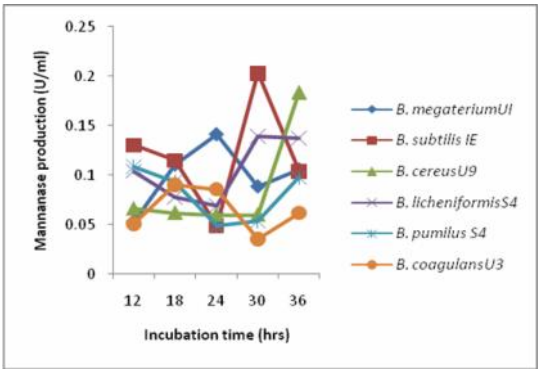

Figure 3b. *Effect of incubation time on mannanase production by the B. strains*

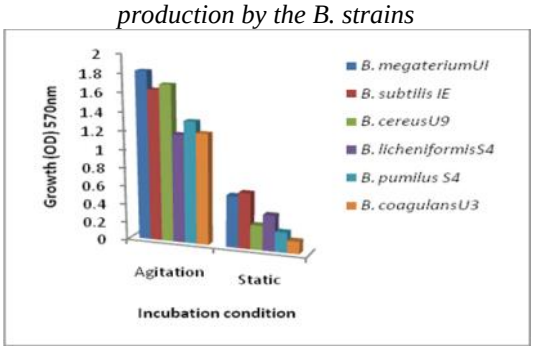

Figure 4a. *Effect of agitation and static conditions on the growth of B. strains*

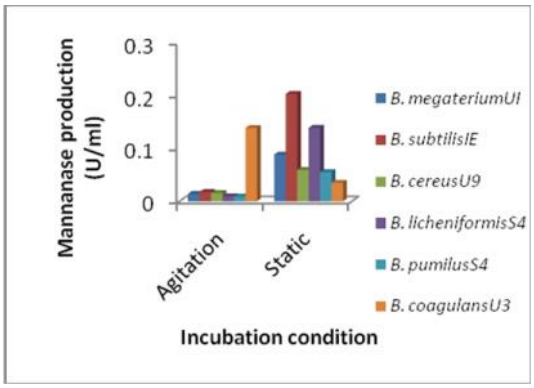

Figure 4b. *Effects of agitation and static conditions on mannanase production by B. strains*

Optimum production of mannanase under agitation condition contrasts the observation from *B. circulans* (Feng *et al.*, 2003) and *Aspergillus niger* (Ab-Rashid *et al.*, 2011). Low production of mannanase by *B. coagulans* during agitation may be due to the aggravation on the organism, caused by the shearing effects of agitation speed. Optimum growth recorded under agitation is agreement with the work of Feng *et al.* (2003).

Carbon source had a significant effect on the growth and mannanase produced by the *Bacillus* strains as shown in Figure 5a and 5b. Among the carbon source used, glucose and fructose was found to induce optimum growth and production respectively. The growth ranged from 0.074f - 0.611a in which *B. licheniformis* S4 had the optimum growth.

Mannanase production ranged from 0.042 - 0.1179U/ml in which *B. subtilis* IE had the optimum production followed by *B. megaterium* UI and *B. pumilus* S5 while the least was observed by *B. coagulans* U3.

Table 5 showed the total protein (mg/ml) produced by the *Bacillus* strains under different carbon source. It ranged from 0.702 to 2.405 mg/ml, in which *B. cereus* had the highest when fructose was used as carbon source.

Nitrogen source had a significant effect on the growth and mannanase produced by the Bacillus strains as shown in Figure 6a and 6b respectively. Among the nitrogen source used, yeast extract and urea was found to be induce optimum growth and production respectively. The growth ranged from 0.225f - 1.561a in which *B. megaterium* UI had the optimum in yeast extract and urea.

Mannanase production ranged from 0.0218f - 0.198a U/ml in which *B. subtilis* IE had the highest. NH_4NO_3 and $(NH_4)_2SO_4$ did not support production of mannanase. Table 6 showed the total protein (mg/ml) produced by the *Bacillus* strains at different nitrogen source. It ranged from 0.05 to 0.460 mg/ml, in which *B. licheniformis* S4 had the highest when yeast extract was used as nitrogen source. Urea induced the optimum production of β-mannanase from *B. suibtilis* IE among the nitrogen source used.

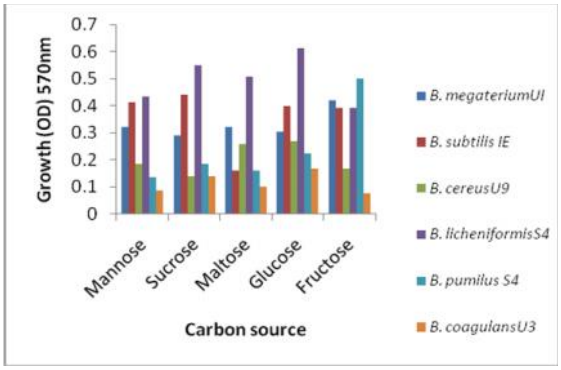

Figure 5a. *Effect of carbon source on the growth of B. strains*

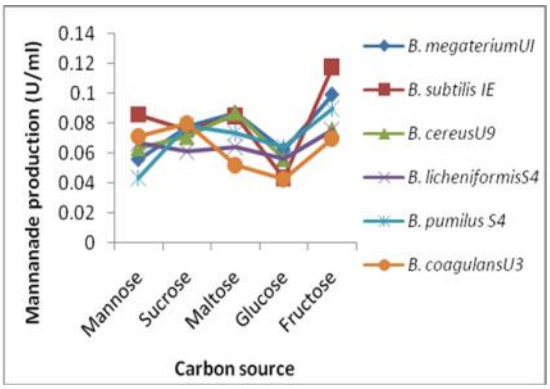

Figure 5b. *Effect of carbon source on mannanase production by B. strains*

Table 5. *Total Protein (mg/ml) produced by the B. strains at different carbon source.*

Isolate	Carbon source				
	Mannose	Sucrose	Maltose	Glucose	Fructose
B. megaterium UI	1.164f	1.426a	1.067d	1.431a	1.565e
B. subtilis IE	1.294d	1.282b	0.778e	1.230d	1.794d
B. cereus U9	1.665a	0.956d	0.702f	1.382c	2.405a
B. licheniformis S4	1.242e	0.816e	1.615a	0.909f	2.257b
B. pumilus S5	1.335c	1.093c	1.370b	1.425b	2.202c
B. coagulans U3	1.423b	1.094c	1.158c	1.177e	0.768f

Data along the same column with different superscript alphabets are significantly different ($P \geq 0.05$)

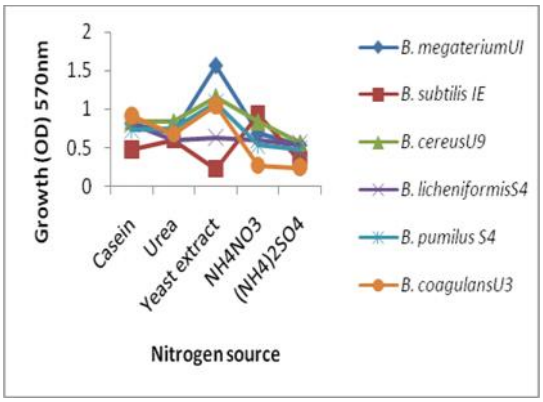

Figure 6a. *Effect of nitrogen source on the growth of B. strains*

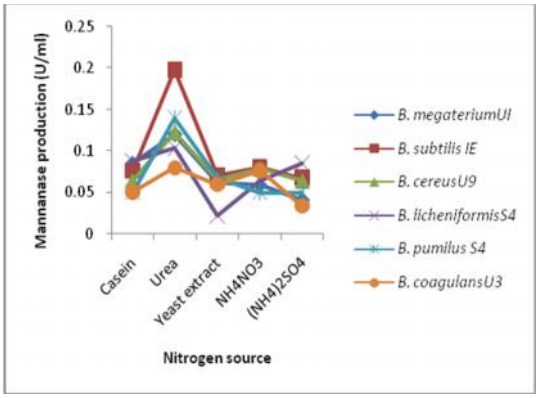

Figure 6b. *Effect of nitrogen source on mannanase production by the B. strains*

Table 6. *Total Protein (mg/ml) produced by the Bacillus strains at different nitrogen source*

Isolate	Nitrogen source				
	Casein	**Urea**	**Yeast extract**	**NH_4NO_3**	**NH_2SO_4**
B. megaterium UI	0.380a	0.180e	0.440c	0.170c	0.430a
B. subtilis IE	0.321c	0.311c	0.300f	0.050f	0.290d
B. cereus U9	0.341b	0.410b	0.310e	0.210a	0.400b
B. licheniformis S4	0.290d	0.220d	0.460a	0.140d	0.390c
B. pumilus S5	0.380a	0.200d	0.390d	0.085e	0.280e
B. coagulans U3	0.220e	0.420a	0.441b	0.190b	0.400b

Data along the same column with different superscript alphabets are significantly different ($P \geq 0.05$)

Carbon source had reasonable effect on mannanase production by the B. strains. This called for a need to supplement Locust Bean Gum (LBG) with other carbon source because LGB are derived from degradation of organic compound which may not be enough to support microbial growth fully (How and Ibrahim, 2004).

From this study fructose served as the best carbon source that favor mannanase production.

Ability of urea to induce the optimum production of β-mannanase from *B. subtilis* IE among the nitrogen source used is contrary to report of Ab-Rashid *et al.* (2011) who reported ammonium nitrate to be the best nitrogen source for *A. niger*.

This may be due to the fact that supplementation of nitrogen source (urea in this case) is important as it plays major role in biosynthesizing cells metabolites and maintains the physiology of the cells. Urea stability makes it suitable for use in agricultural waste (lingo-cellulose) bio-degradation where its concentration is often high because it is used as organic fertilizer (Ab-Rashid *et al*., 2011).

From this study it can be concluded that static condition, fructose, urea, 40^{o}C, pH 5 and 30 hours of incubation time favored mannanase production while agitation condition, glucose, yeast extract, 40^{o}C, pH 3 and 36 hours of incubation time favored the growth of the *B. strains*.

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