



Production, Optimization, Purification and Characterization of Mannanase Produced by *Bacillus Subtilis*

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Authors' contributions

This work was carried out in collaboration between both authors. Author ATBC designed the study, managed the analysis of the study and the literature searches while author ODA performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and also managed the analysis of the study and literature searches. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMB/2020/v20i630254

Editor(s):

(1) Dr. Muhsin Jamal, Abdul Wali Khan University, Pakistan.

Reviewers:

(1) B. Sathya Priya, Bharathiar University, India.

(2) Jorge Serment-Guerrero, Instituto Nacional de Investigaciones Nucleares, México.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/57367>

Original Research Article

Received 14 April 2020

Accepted 19 June 2020

Published 13 July 2020

ABSTRACT

Endo- β -1,4-D-mannanase (β -mannanase; EC 3.2.1.78) catalyses the random hydrolysis of mannoglycosidic bonds in mannan-based polysaccharides. These enzymes are commonly found in nature and are located within the structure of mannans and heteromannans (galactomannan, glucomannan and galactoglucomannan) in the hemicellulose fraction of trees with soft tissues and hard tissues, locust bean seed. Most β -mannanases degrade manno oligosaccharides down to a degree of polymerization of four. In this study mannanase was produced using a submerged fermentation method from *Bacillus subtilis*. The effect of growth of the organism and processing parameters on the production of mannanase were determined after which optimization studies were carried out. The enzyme was partially purified using ammonium sulphate, dialysis and gel filtration. The partially purified enzyme was characterized. The result of the study showed that mannanase enzyme from *Bacillus subtilis* was optimally produced in a medium comprising of galactose, peptone, sugarcane bagasse at a pH of 6.0 and a temperature of 45 oC. The enzyme was most stable and active at pH 10.0 and at a temperature of 40 oC. and 60 respectively.

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Keywords: *Glucomannan; galactomannan; galactoglucomannan; mannoooligosacharride.*

1. INTRODUCTION

Lignocellulose is a major component of plant cell walls and is mainly composed of lignin, cellulose and hemicellulose. Hemicellulose is the second most abundant polysaccharide found in nature and mannan is one of its primary constituents. Mannan, which forms one of the major constituent groups of hemicelluloses in the wall of higher plants, is composed of linear or branched polymers derived from hexose sugars such as D mannose, D-glucose and D galactose, from pentoses such as D-xylose, L-arabinose and also from sugar acids. These are linked together by β -1,4-glycosidic bonds but other bonds such as β -1,3-, β 1,6-, α -1,3-, α -1,6-glycosidic bonds have also been reported [1]. Hemicelluloses are composed of both five carbon and six carbon sugars. They are irregular in structure and are more prone to hydrolytic reactions. Within biomass, the hemicelluloses are situated between the lignin and the collection of cellulose fibres underneath [2].

Mannans and heteromannans are a part of the hemicellulose fraction in plant cell walls. The structure of hemicellulose is a key component of many types of sugar such as xylans, mannans, heteromannans, galactans, and arabinans . Mannans and heteromannans are widely distributed in hardwoods and softwoods, seeds of leguminous plants, and bean. They can be divided into 4 types: (1) unsubstituted (1,4)-mannans, (2) galactomannans, (3) glucomannans, and (4) galactoglucomannans [3].

Mannans are ubiquitous and occur as constituents of the hemicellulose in woods, in nature as part of the hemicellulose fraction in hardwoods and softwoods, seeds of legumes and in beans.

Mannan acts as a cell wall structural component in algae and as a storage carbohydrate in bulbs and plant endosperms. It also provides mechanical resistance in seeds and beans. In addition, mannan is a major component of the hemicellulose fraction in soft woods. It can be degraded to mannose by β -mannanases, and these enzymes are usually produced by plants, bacteria, and fungi.

Mannanase (1, 4- β -D-mannan mannanohydrolase; 3.2.1.78) is the enzyme that

cleaves the β -1, 4-mannosidic linkages in mannans, galactomannans, glucomannans, and galactoglucomannans.

They are hydrolytic enzymes which hydrolyze randomly 1,4 mannosidic linkages within the backbones of mannan, galactomannan, glucomannan and galactoglucomannan [3], they attack the internal glycosidic bonds of mannan backbone to release the condensed β -1,4-manno-oligosaccharides.

Mannanases are ubiquitous in nature and are elaborated by a compendia of microorganisms largely isolated from natural environments. β -mannanases have been isolated and characterized from various fungi bacteria [4], plant and animals . β -mannanases from bacteria include sources such as aerobes, anaerobes and extremophiles (thermophiles, halophiles and psychrophiles). β mannanases are mostly produced extracellularly, but cell wall bound mannanases have also been reported. β mannanases from plant origin are involved in seed germination and fruit ripening.

Although multiple β -mannanase-producing bacteria have been reported, they are far from the diverse industry needs. Currently, acidic and alkaline β -mannanase have been proposed to meet the industrial demands.

β -mannanase has a great potential in many industrial applications including foods, feed for animals and plants, pulp and paper, pharmaceuticals and cosmetics, the production of mannan and manooligosaccharids, bioethanol and biodiesel productions, and oil and textile industries. Mannanases are useful in many fields including biobleaching of pulp and detergent industry, (bioconversion of biomass wastes to fermentable sugars and upgrading of animal feed stuff. It can be used to reduce the viscosity of coffee extracts. The coffee preparation using β -mannanases showed better volatile aroma, taste properties and visual appearance of the final drink. Mannanases could be used as valuable food sweetener sweetener or additives and also have potential application for mannoooligosaccharide preparation to be used as prebiotic, which is expected to improve the growth performance of animal. In this study, β -1,4-mannanase enzyme produced extracellularly from the *B. subtilis* that was isolated from the soil was purified, and characterized.

2. MATERIALS AND METHODS

2.1 Culture Collection

2.1.1 Microorganism and cultivation

Bacillus subtilis was obtained from the culture collection of our previous work in the Industrial and Biotechnology unit of the Department of Microbiology, University of Ibadan, Ibadan Nigeria.

2.1.2 Morphological identification

The selected isolate was examined for cell shape, Grams staining, spore staining, motility test, oxidase, catalase, citrate, indole, urease, starch hydrolysis and sugar utilization.

2.1.3 Molecular identification of the Isolate

The sequencing of 16SrRNA sequence of the isolates was compared with other bacterial sequences deposited in the GenBank database using the BLAST algorithm. The results showed that both 16S rRNA sequence of the isolate was identified to *Bacillus subtilis*, with the level of identity of 99%

2.1.4 Preliminary screening of the isolates for Mannanase production on solid agar

Preliminary screening for 1, 4 β -Mannanase production was done using modified Mineral Salt Agar Medium (MSA) (30) containing (g/l) galactomanan 10, NH_4NO_3 0.3, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.05, K_2HPO_4 7.54, KH_2PO_4 2.32, agar 20, pH7.0. The medium was sterilized and the agar plates were inoculated by inserting a cork borer into an actively growing bacterial colony on Nutrient Agar and incubated for 24 h at 35°C. Mannanase activity was detected on the cultures by staining the plates with iodine solution for 15min. Galactoman hydrolysis was observed by the appearance of clearing zones around the bacterial colony.

2.1.5 Production of mannanase using submerged fermentation

The modified MSM mineral salts medium was used for enzyme production. The initial pH was adjusted to 7 by HCl or NaOH. Erlenmeyer flasks (100 ml) containing 20 ml sterile mineral basal medium (MBM) were inoculated with 0.6 ml seed culture (O.D=1) exponentially growing on LB

medium. Submerged batch culture was carried out with agitation (150 rpm) at 35°C for 24 hr.

2.1.6 Determination of enzyme activity

Mannanase was assayed by measuring the reducing sugars using Dinitrosalicylic Acid (DNS) method [5]. The mannanase assay mixture contained 0.5 ml of 0.5% (w/v) locust bean gum (substrate), prepared in 50 mM phosphate buffer, pH 7 and 0.5ml of appropriately diluted culture broth. The reaction mixture was maintained at 50°C for 30 min.

After incubation 1 ml of DNS Reagent was added and boiling took place from 5 – 15 min. The developed red brown color was measured at 575 nm. One unit of enzyme activity (U) was defined as the amount of enzyme liberating 1mol of mannose per minute under the assay conditions. Specific activity was expressed as U/mg of protein. Controls were routinely included in which enzyme preparation or substrate was omitted.

2.1.7 Protein determination

Soluble protein concentration was measured by the method of Lowry *et al.*, [6] using bovine serum albumin as standard.

2.1.8 Partial purification of β -mannanase

The β mannanase was partially purified using ammonium sulphate, dialysis and gel filtration. The purification steps were carried out at 4°C. The cell free crude enzyme filtrates were precipitated using ammonium sulphate. The precipitates were collected by centrifugation at 8000 rpm at 4°C for 15 min and resuspended in Sodium citrate buffer (0.1 M) pH 5.6. The precipitate was further purified using dialysis tube and sephadex.

2.1.9 Effect of various production parameters on the growth and mannanase production

The effect of carbon sources (glucose, sucrose, lactose, galactose, maltose, mannose), nitrogen (peptone, yeast extract, sodium nitrate, ammonium chloride, and ammonium sulphate), cheap agricultural residues (coconut guar, bean pod, copra meal, sugarcane bagasse and groundnut peel), pH (3.0 - 10.0) and temperature (30°C – 60°C) on the growth and mannanase production by *Bacillus subtilis* was investigated. The production medium was supplemented with

1% of the substrates. The supplemented medium was sterilized, inoculated and incubated at 35°C for 24, 48 and 72 hours. After incubation the fermentation medium was analysed for growth and enzyme production.

2.2 Characterisation of the β -Mannanase Produced by *B. subtilis*

2.2.1 Effect of temperature on enzyme activity and stability

The effect of temperature (25, 28, 30, 35, 40 and 44°C) and pH (4, 4.5, 5, 5.5, 6 and 6.5) on enzyme activity and stability was determined. 0.3 ml of enzyme was added to 2.7ml of 0.5% LBG in 0.1M sodium citrate buffer (pH 5.6). The reaction mixture was incubated at for 30 min. Mannanase activity was determined according to the method of Miller et al. [5].

2.2.2 Effect of metal ions on enzyme activity

The effect of metal ions and substrate concentration on mannanase activity was determined. The reaction mixture containing 0.5ml of the enzyme with 0.5ml of locust bean gum containing 1mM of Ca^{2+} , Cu^{2+} , Co^{2+} , Hg^{2+} and Mn^{2+} in 50Mm citrate buffer (pH 6.0) at 50°C for 30min, mannanase activity was determined using the method of Miller et al. [5].

2.2.3 Effect of substrate concentration

The effect of substrate concentration on enzyme activity was determined. Different concentration of locust bean gum (0.25-1.0%) was dissolved in 0.1M citrate phosphate buffer. 1ml of the solutions was added to 1ml enzyme samples and incubated for the 30 min at 50°C. The reducing sugar released was monitored using the DNS procedure [5]. A reciprocal quantity of reducing sugar recorded was plotted against a reciprocal of the substrate concentration. The V_{max} , K_{max} and the regression equation were determined from the line equation obtained.

2.2.4 Purification of mannanase

Mannanase purification steps were sequentially carried out using Ammonium sulphate, enzyme dialysis and column chromatography.

2.3 Statistical Analysis

Statistical analysis was carried out on results obtained in this work using one-way Anova test

while means were separated using Duncan's Multiple Range Test.

3. RESULTS

3.1 Growth and Mannanase Production

The highest growth rate for *Bacillus subtilis* was recorded at the 48 hours of incubation with a range of 0.6 – 0.8g/L while mannanase enzyme production ranged from 2.57 to 6.37U/ml. however it was observed that at the 72 hours of fermentation mannanase production reached its maximum as can be shown in Fig. 1.

3.2 Effect of Carbon (Simple Sugars) on Growth and Mannanase Production

As shown in Fig. 2a and 2b, the best carbon source supporting the growth of *Bacillus subtilis* is mannose while galactose supported the maximum production of mannanase. The growth ranged from 0.453 - 0.894OD while the mannanase production ranged from 5.18 - 5.85 U/MI.

3.3 Effect of Nitrogen Sources on Growth and Mannanase Production

The effect of nitrogen sources on the growth and mannanase production by *Bacillus subtilis* is shown in Table 1. The growth ranged from 0.608 - 1.507 OD. While its mannanase production ranged from 2.91 - 5.60 U/mL Peptone supported the highest growth of *Bacillus subtilis* and its mannanase production.

3.4 Effect of Agricultural Waste on Growth and Mannanase Production

Sugar cane bagasse supported the highest growth rate of *Bacillus subtilis* and its mannanase production as shown in Table 2. The growth ranged from 0.377 - 1.722 OD while its mannanase production ranged from 2.36 to 5.34 U/mL.

3.5 Effect of Carbon (Simple Sugars) on Growth and Mannanase Production

The effect of carbon sources on growth and mannanase production by *Bacillus subtilis* is shown in Fig. 2a and 2b. The growth ranged from 0.453 - 0.894OD. Mannose supported the highest growth at the 72 hrs. The mannanase

production ranged from 5.18 - 5.85 U/mL. Galactose supported the highest mannanase production by *Bacillus subtilis*. The ability of galactose to support the highest mannanase production could be attributed to the ease of accessibility of the simple sugar by the strain [3,7]. In a similar work

Shanty *et al.*, [8] reported the enhancement of mannanase production by simple sugars such as glucose and fructose in *Nonomuraea* sp. while Adebayo Tayo *et al.*, [9] reported the use of fructose as the best carbon source supporting maximum mannanase production by *Bacillus* strains.

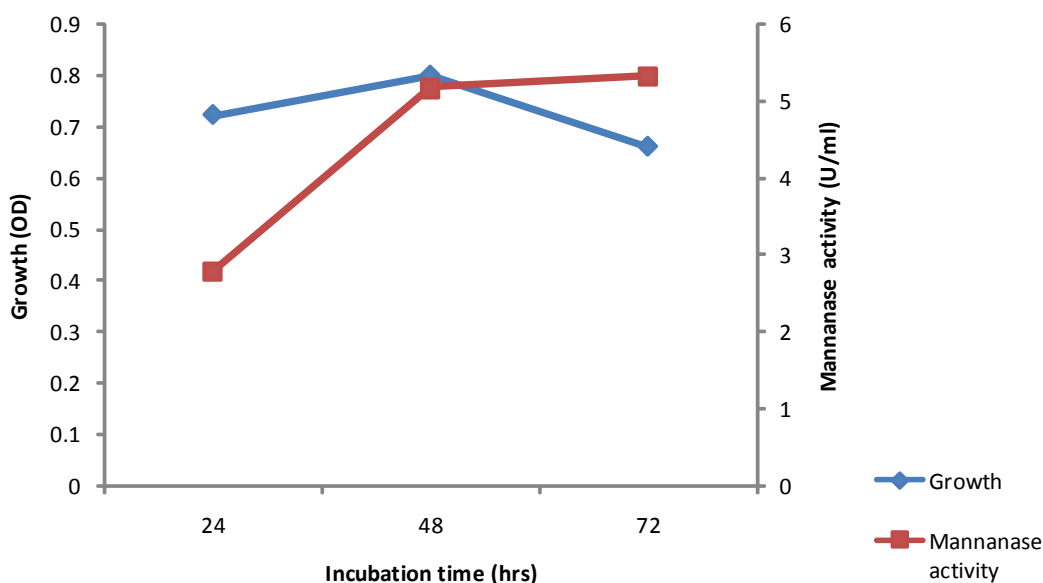


Fig. 1. Growth and Mannanase production by *Bacillus subtilis* in submerged fermentation

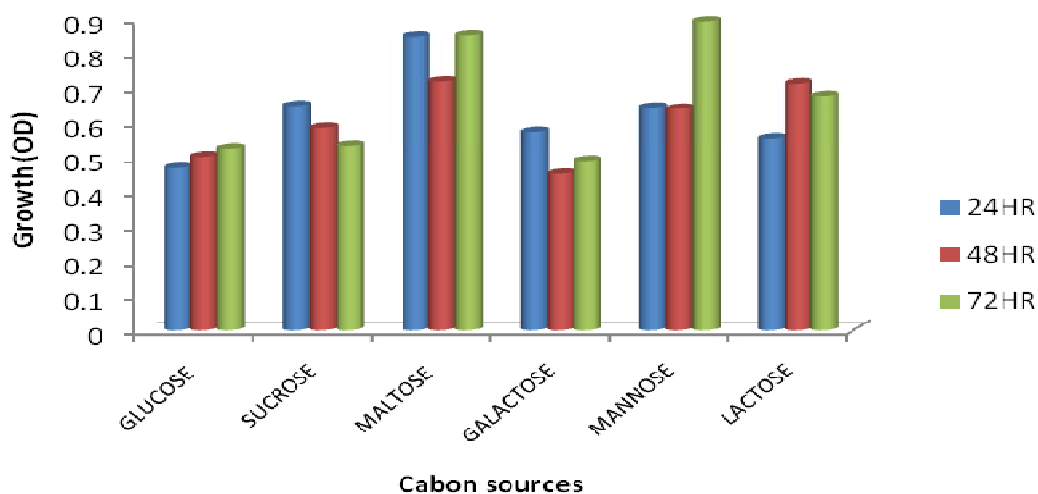


Fig. 2a. Effect of carbon sources on growth of *Bacillus subtilis*

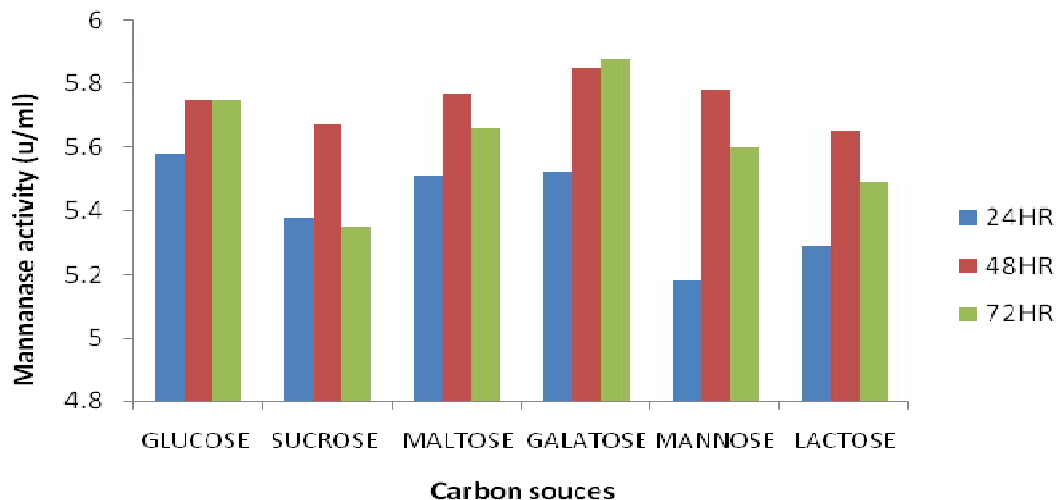


Fig. 2b. Effect of carbon sources (Simple sugars) on mannanase production by *Bacillus subtilis*

Table 1a. Effect of nitrogen sources on growth of *Bacillus subtilis*

Nitrogen sources	Growth (OD)/ Incubation (hrs)		
	24HR	48HR	72HR
Yeast extract	1.40±0.070 ^b	1.494±0.06 ^b	1.357±0.04 ^b
Peptone	1.433±0.01 ^a	1.507±0.00 ^a	1.427±0.00 ^a
Ammonium chloride	1.071±0.21 ^c	0.948±0.22 ^d	1.061±0.23 ^d
Ammonium sulphate	1.078±0.31 ^c	1.098±0.32 ^c	1.161±0.30 ^c
Sodium nitrate	0.726±0.01 ^d	0.608±0.02 ^e	0.713±0.00 ^e

Data are means of three replicates. Means with different letters within each column differ significantly ($P \leq 0.05$)

Table 1b. Effect of nitrogen sources on mannanase production by *Bacillus subtilis*

Nitrogen sources	24hr	48hr	72hr
Yeast Extract	3.99±0.00 ^a	5.55±0.01 ^a	5.08±0.01 ^b
Peptone	2.91±0.02 ^e	5.6±0.01 ^a	4.88±0.01 ^c
Ammonium chloride	3.51±0.01 ^c	5.07±0.00 ^c	5.08±0.01 ^b
Ammonium sulphate	3.72±0.12 ^b	5.5±0.10 ^a	5.35±0.11 ^a
Sodium nitrate	3.35±0.21 ^d	5.39±0.20 ^b	4.41±0.21 ^e

Table 2a. Effect of agricultural waste on growth of *B. subtilis*

Agricultural residue	Time		
	24hr	48hr	72hr
Groundnut peel	1.051±0.00 ^c	1.121±0.00 ^d	1.318±0.00 ^b
Sugarcane bagasse	1.057±0.00 ^c	1.720±0.01 ^a	1.118±0.00 ^c
Copra meal	0.377±0.00 ^d	1.41±0.01 ^b	0.939±0.00 ^e
Bean peel	1.240±0.01 ^b	0.878±0.00 ^e	1.006±0.00 ^d
Coconut guar	1.280±0.01 ^a	1.192±0.00 ^c	1.476±0.00 ^a

Data are means of three replicates. Means with different letters within each column differ significantly ($P \leq 0.05$)

3.6 Effect of Nitrogen Sources on Growth and Mannanase Production

The effect of nitrogen sources on the growth and mannanase production by *Bacillus subtilis* is shown in Table 1. The growth ranged from 0.608 - 1.507 OD. The highest growth was recorded in peptone while the least reading was found in sodium nitrate. Mannanase production ranged from 2.91 - 5.60 U/mL. Peptone supported the highest production. The nitrogen source can significantly affect the pH of the medium during the course of fermentation. [10]. The ability of peptone to support the highest mannanase yield by *Bacillus subtilis* may be due to the fact that peptone as an organic nitrogen source is not involved in the competition with active site of the mannanase and moreover organic nitrogen source are preferred as they are cheaper in cost [11]. Similar results have been reported by Puchart *et al.*, [12] by *Bacillus nealsonii* PN-11 where organic nitrogen sources *viz*, tryptone, casein, bactopectone, beef extract and yeast extract supported higher mannanase yield than the inorganic nitrogen sources (The effect of organic nitrogen sources on mannanase production have been reported by (11,55) in various *Bacillus* strain *viz*. *Bacillus polymyxa*, *Bacillus subtilis*, *Bacillus brevis*) [13] reported that beef extract and peptone supported mannanase production by *Clostridium tertani*. Lin *et al.*, [14] reported the use of yeast extract for maximum mannanase production by *Bacillus* sp. Shanti *et al.*, [8] reported the use of malt extract as best nitrogen source for mannanase production by *Nonomuraea* sp. Soybean meal an organic nitrogen source was found to give the maximum production of mannanase in *Penicillium italicum* as reported by Akinyele *et al.*, [15]. Zhang *et al.*, [16] reported polypeptone as the best nitrogen source supporting mannanase production in *Bacillus* sp. while Adebayo- Tayo *et al.*, [17] reported urea as the best nitrogen

source supporting mannanase production by *Bacillus subtilis*.

3.7 Effect of Agricultural Waste on Growth and Mannanase Production

The effect of agrowaste on growth and mannanase production by *Bacillus subtilis* is shown in Table 2. The growth ranged from 0.377 - 1.722 OD. The highest growth was recorded when sugarcane bagasse was used while the least was recorded when copra meal was used. The mannanase production ranged from 2.36 to 5.34 U/mL. Sugarcane bagasse supported the highest production of mannanase while coconut guar supported the least. The use of agro industrial waste has been used by many researchers as source of carbon for mannanase production mainly due to substrate cost and availability. The *Bacillus* strain grew well on various raw materials of commercial potential importance with significant differences in the rate of mannanase production. The occurrence of variation in mannanase yield may be due to the nature of cellulose or hemicellulose, presence of some components (activators or inhibitors) in these materials and variations in the substrate accessibility [18].

3.8 Effect of pH on Growth and Mannanase Production

The effect of pH on the growth and mannanase production by *Bacillus subtilis* is shown in Fig. 3. The growth ranged from 0.458 - 1.448OD. The highest value was recorded at pH 6.0 while the least was recorded at pH4.0. The mannanase production ranged from 3.45 - 5.89 U/ml. The highest value was recorded at pH 6.0 while the least was recorded at pH 3.0. Microorganisms are very sensitive to pH. Therefore, the selection of optimum pH is very essential for the production of enzymes [19].

Table 2b. Effect of agricultural waste on mannanase production by *B. subtilis*

Agricultural waste	Time		
	24hr	48hr	72hr
Groundnut peel	5.25±0.01 ^a	5.12±0.01 ^b	2.57±0.01 ^d
Sugarcane bagasse	3.5±0.07 ^b	5.34±0.05 ^a	4.4±0.07 ^a
Copra meal	3.0±0.04 ^c	4.3±0.07 ^c	2.69±0.06 ^c
Bean peel	2.99±0.01 ^d	5.2±0.04 ^a	3.03±0.03 ^b
Coconut guar	3.06±0.02 ^c	5.32±0.01 ^a	2.36±0.03 ^e

Data are means of three replicates. Means with different letters within each column differ significantly ($P \leq 0.05$)

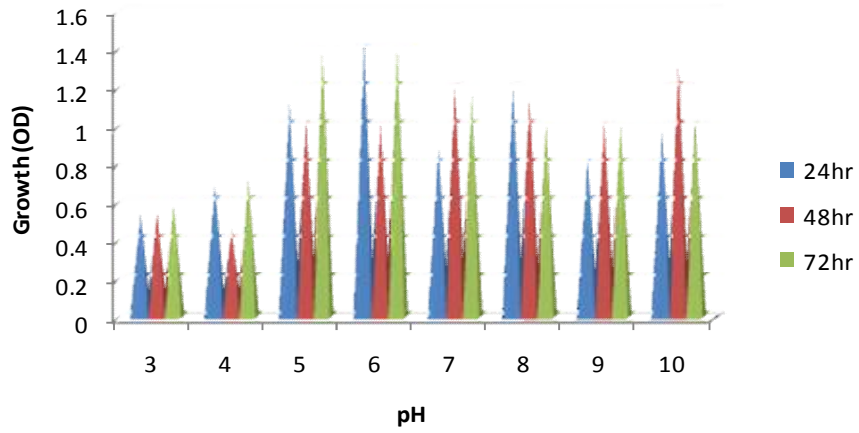


Fig. 3a. Effect of pH on the growth of *B. subtilis*

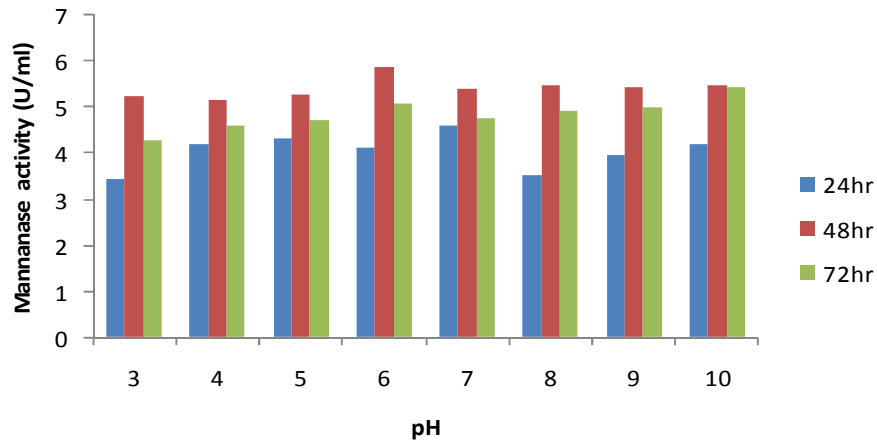


Fig. 3b. Effect of pH on mannanase production by *Bacillus subtilis*

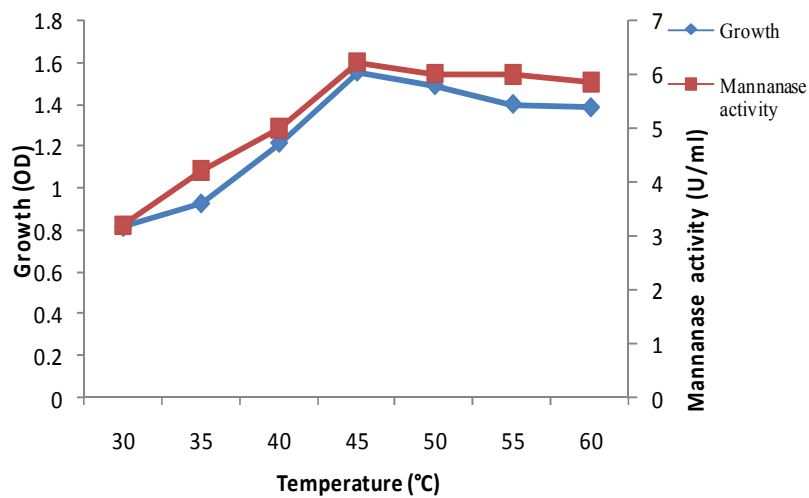


Fig. 4. Effect of temperature on growth and mannanase production

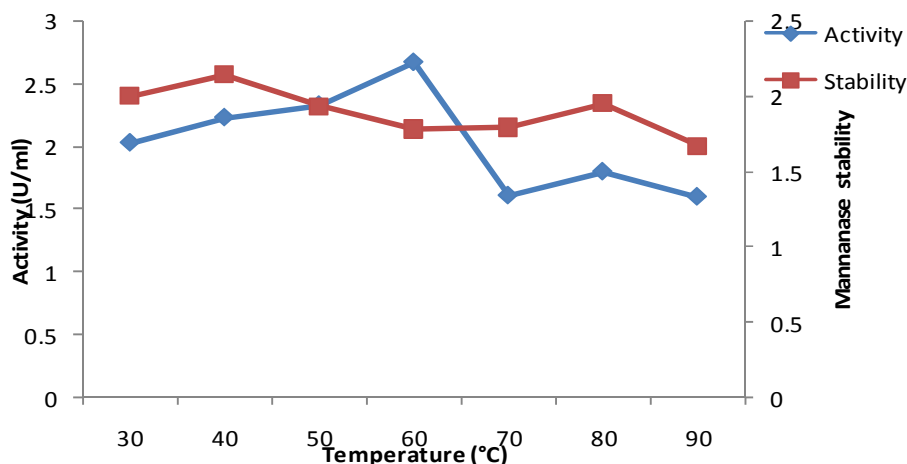


Fig. 5a. Effect of temperature on activity and stability of mannase

3.9 Effect of Temperature on Growth and Mannanase Production

The effect of temperature on the growth and mannase production by *Bacillus subtilis* is shown in Fig. 4. The growth ranged from 0.812 to 1.555OD. The highest growth was recorded at 45°C while the least was recorded at 30°C. The mannase production ranged from 3.21 to 6.21U/mL. The highest was recorded at 45°C while the least was recorded at 30°C. The temperature of the fermentation medium is one of the vital factors having deep influence on the end products [20]. The optimum temperature for mannase production in *Bacillus subtilis* was found to be 45°C. Ability of mannase to be produced at this temperature is due to the fact that the optimum temperature for mannase production often corresponds with the growth temperature of the respective microorganisms. This is in agreement with Khampheng, [21] who reported a temperature of 45°C for mannase production from *Bacillus circulans* and Khanongnuch *et al.*, [22] reported a temperature of 45°C for mannase production from *Bacillus subtilis* 5H. However this is in contrast to Abe *et al.*, [17] who reported an optimum temperature of 60°C for mannase production from *Bacillus* sp kk01 and Ferreira and Filho [23] reported an optimum temperature of 55°C for mannase production from *Trichoderma harzianun*.

3.10 Effect of Temperature on Mannanase Activity and Stability

The effect of temperature on *Bacillus subtilis* mannase activity and stability is shown in Fig. 5a. The mannase activity ranged from 1.60 to

2.67U/ml. The highest mannase activity was recorded at 60°C while the least activity was recorded at 90°C. The stability ranged from 1.67 to 2.00U/ml. The highest stability was recorded at 40°C while the least was recorded at 90°C. Generally, bacteria mannase are optimally active at 50-60°C. This is similar to the report of Rammanagari *et al.*, (2011) who reported a mannase activity of 60°C for *Paenibacillus* sp. and Meenakshi *et al.*, [24] reported a mannase activity at 65°C in the solid state fermentation and characterization of partially purified thermostable mannase from *Bacillus* sp. In contrast mannase activity of 50-55°C was reported by Mendoza *et al.*, [25] from *B. subtilis* strains NM-39, Khanongnuch *et al.*, [22] from *B. subtilis* strains 5H, from alkaliphilic *Bacillus* sp. StrainJAMB-750.

3.11 Effect of pH on Activity and Stability of Mannanase Produced by *B. subtilis*

The effect of pH on mannase activity and stability is shown in Fig. 5b. The mannase activity ranged from 1.50 to 2.26 U/ml. The highest activity was recorded at pH 10.0 while the least was recorded at pH3.0. Mannanase stability, ranged from 1.04 to 2.04U/ml. The highest stability was recorded at pH 10.0 while the least was recorded at pH 6.0. This is similar to Akino *et al.*, [4] reported a mannase from alkalophilic *Bacillus* with pH of 9.0, however this is in contrast with Zhang *et al.*, [26], Zakaria *et al.*, [27] and Ooi and Kikuchi [28] who reported a mannase activity at pH 7.0 from *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus* sp. respectively. pH 7.5 for mannase activity in *Streptomyces lipomoea*.

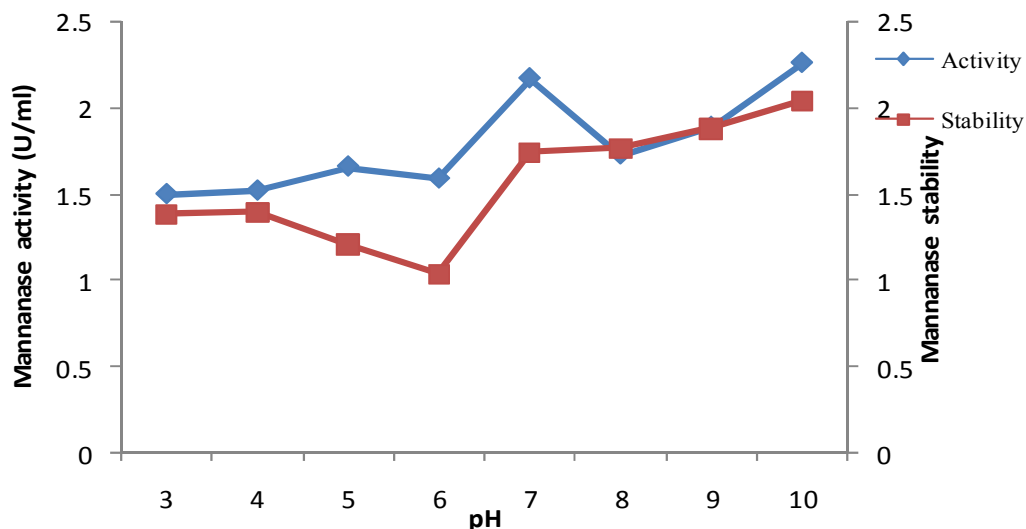


Fig. 5b. Effect of pH on activity and stability of mannanase produced by *Bacillus*. sp

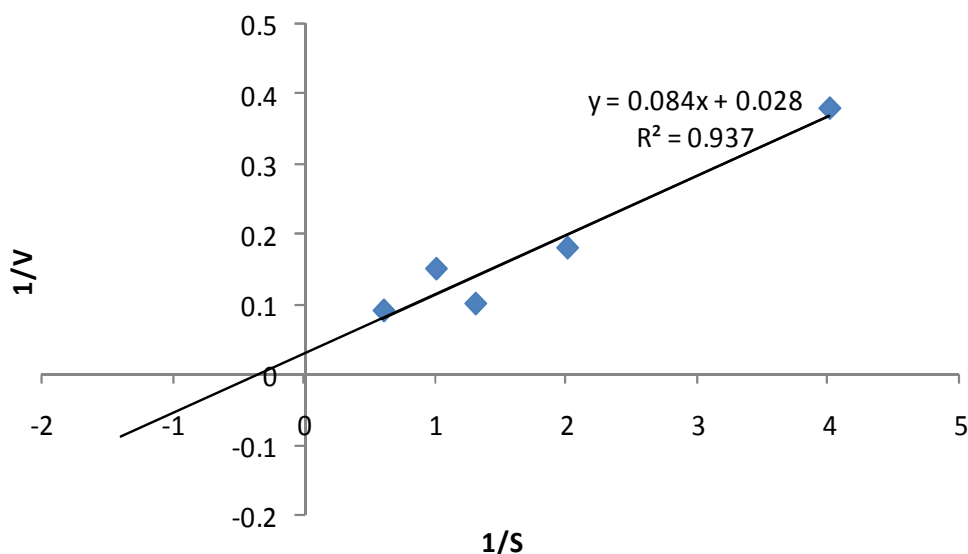


Fig. 6. Lineweaver-Burk plot of the enzymatic activity of *B. subtilis* mannanase at different concentration

The K_m and V_{max} of endo-mannanases are reported to be in the range of 0.0095-27.4 mg/ml and 29-3330 $\mu\text{mol/ml}$ or mg/min, respectively. Locust bean gum and Konjac mannan are the most commonly used substrates for estimation of kinetic parameters. In this study a K_m value of 3.33 mg/ml and V_{max} value of 33.33 $\mu\text{mol/ml}$ was recorded. In a study of mannanase from *Bacillus circulans* reported by Mou *et al.*, [29] a K_m value of 1.26 mg/ml and V_{max} of 30.4

$\mu\text{mol/ml}$ was reported. Furthermore Liu *et al.*, [30] reported a K_m value of 30 mg/ml and V_{max} of 16666.7 $\mu\text{mol/ml}$ in mannanase from *Bacillus subtilis*, Chandra *et al.*, [31] reported a mannanase from *Paenibacillus* with a K_m and V_{max} of 1.05 mg/ml and 714 $\mu\text{mol/ml}$ respectively. Zang *et al.*, [26] reported a mannanase from *Bacillus pumilus* with K_m and V_{max} of 2.14 mg/ml and 2777.8 $\mu\text{mol/ml}$ respectively.

Table 3. Purification properties of mannanase from *Bacillus subtilis*

Form of Mannanase	of	Total volume (ml)	Protein Content	Total protein (mg)	Mannanase activity	Total activity	Specific mannanase activity	Purification fold	% yield
Crude		1000	1.5±0.02 ^a	1500±0.1 ^a	4.74±0.02 ^a	4740±0.31 ^a	3.16±0.02 ^d	1±0.01 ^d	100±0.1 ^a
Ammonium sulphate		100	0.53±0.01 ^b	53±0.15 ^b	3.24±0.00 ^b	324±0.20 ^b	6.11±0.01 ^c	1.93±0.0 ^c	6.83±0.2 ^b
Dialysis		20	0.48±0.00 ^c	9.6±0.2 ^c	3.01±0.01 ^c	60.2±0.21 ^c	6.27±0.00 ^b	1.98±0.02 ^b	1.27±0.0 ^c
Gel filtration		10	0.12±0.03 ^d	1.2±0.0 ^d	2.1±0.01 ^d	21±0.0 ^d	17.5±0.02 ^a	5.53±0.04 ^a	0.44±0.0 ^d

3.12 Purification Properties of Mannanase

The purification properties of mannanase is shown in Table 3. In a three step purification (ammonium sulphate, dialysis and gel filtration) of mannanase, a 0.44% yield, 5.53 purification fold and 17.5IU/mg specific activity was recorded. The purification yield, fold-purification, and specific activity of purified endo-mannanases obtained from different sources are quite variable. Zhang *et al.*, [32] reported purification of endo-mannanase from *Bacillus* sp. MSJ-5 using a combination of salt precipitation, ion exchange, and gel filtration chromatography with an overall yield, fold-purification, and specific activity of 18.9%, 19-fold, and 5383 IU/mg, respectively. Endo-mannanase from *Bacillus subtilis* WY 34 was purified with 20.3% yield, 5.4-fold purification, and 8302 IU/mg specific activity, using a combination of salt precipitation, gel filtration, and ion exchange chromatography [33]. Very low yield of purified endo mannanase (0.8%) was obtained after subjecting crude extract of *Lilium testaceum* to sequential DEAE-Sephacel and Superose12 chromatography [34]. Zhang *et al.*, [32] reported a 47% yield, 33.1 purification fold and specific activity of 4341IU/mg in a four step mannanase purification from *Bacillus licheniformis*, Chauhan *et al.*, [35] reported a 8.92% yield, 38.96 purification fold and specific activity of 2280.9IU/mg in a three step mannanase purification from *Bacillus nealsonii*.

3.13 Effect of Metal Ions on Mannanase Activity of *Bacillus subtilis*

Divalent cations have been shown to stimulate or inhibit enzyme production in microorganisms. In this work Manganese Chloride is shown to have induced maximum activity by *Bacillus subtilis* while it is strongly inhibited by mercury salt. Yu *et al.*, [36], Zakaria *et al.*, [27], Li *et al.*, [37] all reported the strong inhibition of mannanase by mercury salt in various species *B. subtilis* SA-22 (*B. subtilis*, Ku-1) and *B. circulans* k-1 respectively. Montiel *et al.* [38] have also reported a complete inhibition of mannanase activity by mercury. Maaenakashi *et al.*, [24] also reported an inhibition in mannanase activity by mercury salt. Nakajima and Matsuura, [39] observed stimulation in mannanase production from *Clostridium* sp. in the presence of Calcium and Magnesium ions.

4. DISCUSSION

Bacillus species are an important source of mannanase enzymes production which have been used for application in industrial such as coffee extraction, animal feed, food and mannooligosaccharides preparation. 18, 28 58 have all reported the production of mannanase from *Bacillus subtilis*, while Zhang *et al.*, [40] have reported the production of mannanase from *Bacillus licheniformis*. Many researchers attempt to find new source of mannanase including bacteria, fungi and plant seed [35,41].

Mannanase production in a SmF process is influenced by physico-chemical and nutritional parameters like incubation time, pH, temperature, Nitrogen content, and Carbon-source [42].

The optimum incubation time for microbial mannanases ranges from as low as 18h to as high as 264h. A prolonged production phase can result in increased productivity of the fermentation process. In this study 48 hours of fermentation supported the maximum production of mannanase production in this specie. Similarly Vijayalaxmi *et al.*, [43] reported a fermentation period of 48 hours for maximum mannanase production in *Bacillus halodurans* while Meenakshi *et al.*, [24] reported an incubation time of 96 hours for the maximum mannanase production in *Bacillus* sp. Titapoka *et al.*, [44] produced maximum mannanase from *Acinetobacter* sp at 24 hours, Chauhan *et al.*, [35] reported highest mannanase production from *Bacillus nealsonii* at 96 hours of incubation.

pH of the fermentation medium is known to play a significant role in the growth of microorganism and consequent mannanase secretion. Most of the mannanases reported from bacterial sources are generally produced in neutral to alkaline pH range while mannanases from fungi like *Aspergillus niger* are optimally produced in acidic pH range [45]. The effect of pH is related to the growth and metabolic activities of the organism. The ability of *Bacillus subtilis* to produce highest quantity of mannanase at pH 6.0 may be attributed to the fact that this same pH supported the best growth of the *Bacillus subtilis* cells in the fermentation medium. This is in line with the report of Chantorn *et al.*, [3], Khampheng, [21], Jiang *et al.*, [33], and who all reported best mannanase production at pH 6.0 in *Bacillus* sp., *Bacillus circulans*, *Bacillus* sp., *Bacillus subtilis* and *Penicillium oxalicum* respectively. However

Meenakshi *et al.*, [24] reported pH of 6.5 for mannanase production in *Bacillus* sp.

Incubation temperature is one of the most important physical parameters which affect microbial growth and productivity. Usually, the optimum temperatures for endo-mannanase production from bacteria fall in the temperature range of 30-50°C. In this work an incubation temperature of 45°C supported the maximum production of mannanase. Similarly Zakaria *et al.*, [27] reported a temperature of 45°C for mannanase production in *Bacillus subtilis*. Lower temperatures such as 30°C have been reported by Rattanasuk and Ketudat-Cains (2009) from *Bacillus* sp. Meenakshi *et al.*, [24] from *Bacillus* sp. However, relatively higher incubation temperature has been reported for endo-mannanase production from some organisms like *Bacillus subtilis* WY34 (50°C) [33], *B. subtilis* BCC41051 (50°C) [41], and *B. stearothersophilus* (55°C).

Selection of an appropriate substrate is pivotal for optimum production of endo-mannanases under SmF as substrate act as the carbon or energy source. Cost and round-the-year availability are important factors in substrate selection. Endo mannanase production from microorganisms has been found inducible in nature by several researchers. Mannan rich substrates like Konjac powder [40], locust bean gum [7], guar gum [29], and carob seed flour have been predominantly used as substrates as well as inducers for enhanced endo-mannanase production from several microorganisms.

Agricultural by-products represent an excellent source for carbon and nitrogen which can be assimilated by microorganisms. Agro-industrial residues are generated in huge quantities, especially in many agriculture-centric countries, and when not properly discharged or used lead to environmental pollution. Therefore, utilization of low-cost agricultural by-products as carbon/nitrogen sources has been emphasized in order to develop economical, environment-friendly, and efficient processes for endo mannanase production.

Besides carbon source, Nitrogen source is also known to greatly influence endo-mannanase production in SmF process. The nitrogen source can significantly affect the pH of the medium during the course of fermentation. [10]. The ability of peptone to support the highest mannanase yield by *Bacillus subtilis* in this

research may be due to the fact that peptone as an organic nitrogen source is not involved in the competition with active site of the mannanase and moreover organic nitrogen source are preferred as they are cheaper in cost [11]. Similar results have been reported by Puchart *et al.*, [12] by *Bacillus nealsonii* PN-11 where organic nitrogen sources viz, tryptone, casein, bacto-peptone, beef extract and yeast extract supported higher mannanase yield than the inorganic nitrogen sources (The effect of organic nitrogen sources on mannanase production have been reported by [46,32] in various *Bacillus* strain viz. *Bacillus polymyxa*, *Bacillus subtilis*, *Bacillus brevis*). [13] reported that beef extract and peptone supported mannanase production by *Clostridium tertani*. Lin *et al.*, [10] reported the use of yeast extract for maximum mannanase production by *Bacillus* sp. Shanti *et al.*, [8] have reported the use of malt extract as best nitrogen source for mannanase production by *Nonomuraea* sp. Soybean meal an organic nitrogen source was found to give the maximum production of mannanase in *Penicillium italicum* as reported by Akinyele *et al.*, [5]. Zhang *et al.*, [40] have reported polypeptone as the best nitrogen source supporting mannanase production in *Bacillus* sp. while Adebayo- Tayo *et al.*, [9] reported urea as the best nitrogen source supporting mannanase production by *Bacillus subtilis*.

5. CONCLUSION

Bacillus subtilis are non pathogenic organisms that have the GRAS (generally regarded as safe) status, they are generally abundant in nature. In this study *Bacillus subtilis* was used for the production of mannanase under a submerged fermentation method and the optimum mannanase production was achieved at 48 hours of fermentation, temperature of 45°C, pH 7.0, peptone, galactose and sugar cane bagasse.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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