



## Characterization of mannanase-producing bacteria from sago hump

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**Abstract** - Sago is a hardwood that can be used as source of fiber hemicellulose mannan. The abundance of mannanase waste is potential for the production of functional foods such as Manno oligosaccharides. Sago hump which is sago processing waste is very potential for growth of microorganisms because it contains lignin, cellulose, starch, minerals and vitamins that can be used as sources of carbon and energy for the growth. In this study, BLS.11-01 and BLS.11-02 isolates have been isolated and characterized from waste of sago hump. Locust bean gum was used as substrate for measuring the activity of mannanase. Both isolates had optimum temperatures at 70°C and 60°C while they remain stable at temperature range of 30°C - 90°C. Mannanase from BLS.11-01 and BLS.11-02 had optimum pH at 6 and 7, respectively, and were also stable in wide pH range of 2-8. Co<sup>2+</sup> was a mannanase activator for both isolates. Cu<sup>2+</sup>, Zn<sup>2+</sup>, Ni<sup>2+</sup>, Ca<sup>2+</sup> and Fe<sup>2+</sup> were mannanase inhibitors for two isolates. Tween 20 (0.5% and 1.0%) and NaCl (0.5% and 1.0%) were chemical reagents that could enhance the activity of BLS.11-01 and BLS.11-02 mannanase isolates. While tween 80 (0.5% and 1.0%), urea (0.5% and 1.0%) and SDS (0.5% and 1.0%) were chemical reagents that were not effective for both isolates mannanase activity.

**Keywords** - Sago; manno oligosaccharides; waste; mannanase.

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### 1. Introduction

Sago (*Metroxylon* spp.) is a tropical plant which is found in almost all parts of Indonesia. Land area of sago plant in Indonesia reached 1.25 million ha and spread throughout regions including Southeast Sulawesi at 30.000 ha; thus it is a great potency as a source of carbohydrates instead of rice for local citizens. However, sago processing also produces various organic wastes such as bark, pulp and hump [45]. Sago hump is a kind of waste which is very potential for the growth of microorganisms due to its lignin, cellulose, starch, minerals and vitamins contents that can be used as source of carbon and energy for the microorganisms growth. Sago is a hardwood which can be used as source of fiber hemicellulose mannan. The utilization of biomass mannan, particularly from waste palm oil and copra meal, is utilized towards fodder with low absorption rate. Mannan is classified into four subfamilies including linear mannan, glucomannan, galactomanan and

galactoglucomannan [13]. Linear mannan and glucomannan have common structures composed from  $\beta$ -1,4 mannose and  $\alpha$ -1,6-D-galactosyl residues [25, 18, 38, 13]. Glucomannan is a residual combination of  $\beta$ -1,4-mannose and  $\beta$ -1,4-glucose, meanwhile galactoglucomannan is composed from  $\alpha$ -1,6-D-galactopyranose (D-glucosyl and D-mannosyl). Mannan structure has been briefly described by Moreira and Filho [32] and Schröder et al. [40]. Mannans consist of mannose molecule linked together to form a polymer.

As waste mannan, the sago allows microbial decomposition of hemicellulose to grow well. Mannolytic bacteria have been isolated from variety of sources including oil waste [46]; activated sludge [8]; decayed konjac [54] and soil [33]. Currently, some microbes have been identified for having mannolytic activity such as *Weissella viridescens* LB37 [3], *Gloeophyllum trabeum* CBS900.73 [49], *Neosartorya fischeri* P1 [51], *Arabidopsis thaliana* [50], *Bacillus nealsonii* [10], *Bacillus subtilis* Bs5

[21], *Bacillus subtilis* NM-39 [30], *Bacillus sp* KK01 [18], *Thermotoga neopolitana* 5068 [15]. Groups of fungi also exhibits mannolytic activity such as *Streptomyces*, *Aspergillus* and *Trichoderma* [52, 27, 10, 14].

$\beta$ -Mannanases (mannan-endo-1,4- $\beta$ -mannosidase, E.C.3.2.1.78) are hydrolytic enzymes which catalyze the random cleavage of  $\beta$ -1,4 mannosidic linkages within the backbones of mannan, galactomannan, glucomannan and galactoglucomannan [34] resulting in various oligosaccharides as a major product. Additionally, mannanase could be utilized in pulp and paper industry [17, 35]; detergent [4]; coffee [55]; animal feeds [26]; slime control agent [37], Pharmaceutical [36] and oil and gas [12, 47].

The abundance of mannan on waste of sago hump provides an opportunity to discover variety of new bacterial isolates. In this study, we conducted the isolation and characterization of bacteria which have mannolytic activity and characterization of mannanase crude including temperature, optimum pH, stability and the influence of metal ions, detergent and denaturant.

## 2. Materials and methods

### 2.1 Materials

The substrates of locust bean gum and mannose were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade and commercially available.

### 2.2 Methods

#### 2.2.1 Sources of microorganisms

Ten bacterial isolates were isolated from waste of sago hump with BLS.11-01 and BLS.11-02 are two isolates having the greatest mannolytic index. Determination of pure isolates of bacteria were started on mannan-containing medium with composition of 0.2% yeast extract, 0.2% tryptone, 0.02%  $MgSO_4$ , 0.14%  $K_2HPO_4$ , 0.1%  $(NH_4)_2SO_4$ , 20% of bacto agar and 0.3% locust bean gum (modified from Sumardi [44]). Mannolytic activity was determined by clear zone based on staining media using 0.1% Congo red. Isolates that have the greatest mannolytic index were used for the production and characterization of mannanase.

#### 2.2.2 Morphological and biochemical determination

Morphological characterization of isolates refers to the method contained on Bergey's Manual of determinative Bacteriology [19] which includes the observation of microscopic and biochemical tests. Biochemical tests include gram staining, citrate utilization, catalase and carbohydrate fermentation.

#### 2.2.3 Production of $\beta$ -mannanase

Enzyme production is conducted by inoculating of 10% starter in 500 mL of production medium. Production medium contains yeast extract (0.35%), tryptone (0.35%),  $MgSO_4$  (0.035%),  $K_2HPO_4$  (0.245%),  $(NH_4)_2SO_4$  (0.175%), NaCl (0.2%) and LBG (0.65%). The fermentation process was conducted in batch system using bioreactor for 24h at 37°C. The optimum enzyme production period was determined using bacterial cell

growth curve, concentration of the enzyme, and the amount of hydrolyzate. Products containing crude enzyme were centrifuged at 15000 rpm for 15 min. Cell-free supernatant is a crude  $\beta$ -mannanase enzyme.

### 2.2.4 Characterization of enzymes

#### 2.2.4.1 Determination of mannanase activities and protein concentration on crude mannanase

Enzyme activity was measured at 70°C for 60 min using reaction mixture of 1 mL enzyme sample, 1 mL substrate containing 0.5% locust bean gum and 1 mL of 50 mM buffer pH 6.0 and pH 7. Furthermore, 2 mL DNS reagent was added as method for determining reducing sugar [31]. One unit of enzyme activity was defined as the amount of enzyme producing 1 mole of mannose per minute under experimental conditions. Protein concentration was determined by the method of Bradford [7] with bovine serum albumin as a standard.

#### 2.2.4.2 Determination of optimum temperature and crude mannanase stability

Determination of the optimum temperature on mannanase activity in carried out using 50 mM citric and phosphate buffers in optimal pH (6 and 7 for BLS.11-01 and BLS.11-02, respectively) in temperature of 30°C-90°C. Thermal stability of the enzyme was determined by incubating the enzymes for 60 minutes at various temperatures in 50 mM citric and phosphate buffers [44]. Then, the residual enzyme activity was measured using the standard assay.

#### 2.2.4.3 Determination of optimum pH and crude mannanase stability

The optimum pH was determined by measuring the activity of mannanase at pH 2.0–8.0 under standard assay conditions using universal buffer. The effect of pH on enzyme stability was determined using the universal buffer over the range of pH 2.0–8.0. After incubating the enzyme solution at various pH values for 24 h and at 4°C without substrate, the residual enzyme activity was measured using the standard assay.

#### 2.2.4.4 Effects of metal ions and chemical reagents on the activity of crude mannanase

The effects of various additives such as metal ions, detergents and denaturants on the activity of BLS.11-01 and BLS.11-02 were examined. Effect of metal ions on the activity of enzyme was carried out by measuring enzyme activity in optimum pH using reaction mixture of 1 mL crude enzyme and 1 mL (5 mM  $CaCl_2$ ,  $ZnCl_2$ ,  $CoCl_2$ ,  $NiCl_2$ ,  $FeCl_2$  and  $CuSO_4$ ) and 1 mL substrat 0.5%. Enzyme stability was investigated with each chemicals in 50 mM citric and phosphate buffer at room temperature for 60 min. The detergents used to test the stability of the enzyme were sodium dodecyl sulfate (SDS: 0.5% and 1.0%), tween 80 (0.5% and 1.0%) and tween 20 (0.5% and 1.0%). Denaturant agents used were NaCl (0.5% and 1%) and urea (0.5% and 1%). Measurement of residual enzyme activity is carried out by following the above procedure.

Table 1. Effect of metal ions on the activity of the crude mannanase.

Treatment	Relative Mannanase Activity (%)	
	BLS.11-01	BLS.11-02
None	100.00 ± 0.13	100.00 ± 0.04
Metal ions (5 mM)		
FeCl <sub>2</sub>	42.77 ± 0.53	51.06 ± 0.06
NiCl <sub>2</sub>	61.90 ± 0.10	77.16 ± 0.52
CuCl <sub>2</sub>	88.75 ± 0.51	78.14 ± 0.02
CoCl <sub>2</sub>	119.61 ± 0.58	112.23 ± 0.57
ZnCl <sub>2</sub>	71.38 ± 1.03	78.79 ± 0.05
CaCl <sub>2</sub>	83.92 ± 0.05	69.17 ± 0.73

### 3. Results and Discussion

#### 3.1 Screening and identification mannanase producing bacteria

The utilized bacteria was selected from isolates that had the greatest mannolytic index. There were ten isolates which were isolated from waste of sago hump and from the tent, it produced two selected isolates namely BLS.11-01 and BLS.11-02 with MI (Mannolitic Index) of 2.3 and 2, respectively. The different results have been reported by Titapoka et al. [46] who had isolated mannanase bacteria from waste oil and produced two isolates i.e. CW1-2 and ST1-1 with the greatest IM having values greater than 3. The different sources of mannanase bacteria have also been reported by Carr et al. [8]; Mou et al. [33] and Oda et al. [54]. Medium screening used LBG 0.3% as inducer mannanase. Clear zone as an indicator of hydrolysis of LBG medium was known after staining using 0.1% Congo red. Morphological and biochemical determination of BLS.11-01 and BLS.11-02 isolates have been conducted based on method of Bergey's Manual of Determinative Bacteriology [19] which include the observation of microscopical and biochemical tests. Biochemical tests include gram staining, utilization of citrate, catalase and carbohydrate fermentation [48].

Gram staining of BLS.11-01 and BLS.11-02 isolates showed that both isolates were gram-positive. While biochemical determination of BLS.11-01 and BLS.11-02 have been reported by Wahyuni et al. [48]. Biochemical character of BLS.11-01 and BLS.11-02 isolates have also been compared with ST1-1 and *Acinetobacter* sp isolates [46]. In this study, all isolates grew well at room temperature. However, isolates ST1-1 grew well at wider temperature range. Different result was occurred on *Acinetobacter* sp isolate that grow well in temperature range of 20-30°C. Isolates of ST1-1 and *Acinetobacter* sp had the same character in the utilization of citrate test and catalase. Test of the fermentation of carbohydrates showed different results indicated by unhydrolyze maltose of ST1-1. Isolation capability gave positive response in glucose and mannose tests indicating that the isolates could produce mannanase [41].

BLS.11-01 and BLS.11-02 as selected isolates were used for the production of mannanase, while LBG was used as inducer. The potency of BLS.11-02 and BLS.11-01 as source of mannanase were demonstrated by their

activity of 0.4851 and 0.7004 U/mL, respectively. The obtained β-mannanase activities (each for BLS.11-01 & BLS.11-02) were much larger compared to the activity of β-mannanase enzyme of *Bacillus* sp. with locust bean gum as mannan source with activity value of 0.001 U/mL as reported by Abe et al. [2]. The higher activity was reported by Alsarrani [1] who found that mannanase activity of extracellular *A. niger*, *A. flavus*, *A. ochraceus* were 2.90 U/mL, 2.54 U/mL, 2.16 U/mL. The difference these results was mainly due to the used microbes that the different microbes would produce different enzyme activity. These two isolates are interesting strains for producing mannanase that can be applied for sago waste degradation to obtain valuable compounds such as MOS or other functional food in the future.

#### 3.2 Effects of pH and temperature

The effects of pH and temperature on mannanase activities were studied using the supernatants of both BLS.11-01 and BLS.11-02 isolates with results shown in Fig. 1. The optimum pH of mannanase activity were determined by conducting the activity assays at 60°C and 70°C using LBG as the substrate and varying the pH between 2-8. The optimum pH of BLS.11-01 and BLS.11-02 mannanase were 6 and 7, respectively, as shown in Fig. 1. Both of these optimum pH were rather narrow. Their mannanase activities decline tremendously at lower or higher pH values. The same optimum pH has been reported by Titapoka et al. [46]; Mabrouk and Ahwany [28]; Mou et al. [33]; Kim et al. [24]; Chandra et al. [9] and Zhang et al. [39]. Meanwhile, Lu et al. [27]; Yoo et al. [52]; Yang et al. [51] and Wang et al. [50] reported different optimum pH at 11, 4, 5.5 and 5.

The pH stability test of mannanase from BLS.11-01 and BLS.11-02 showed the same stability at wide pH range of 2-8. Both mannanase isolates have greater relative activity of 80%. Both isolates showed stable activity in the area of acids and bases. Mannanase from ST1-1 and *Penicillium occitanis* Pol.6 isolates reported by Titapoka et al. [46] and Blibech et al. [5, 6] possessed stability at wide region of 3-10 and 4-10, respectively. The different of optimum pH of enzyme from various types of microorganisms showed that any microorganism have the properties and characteristics that vary in function of the catalyst.

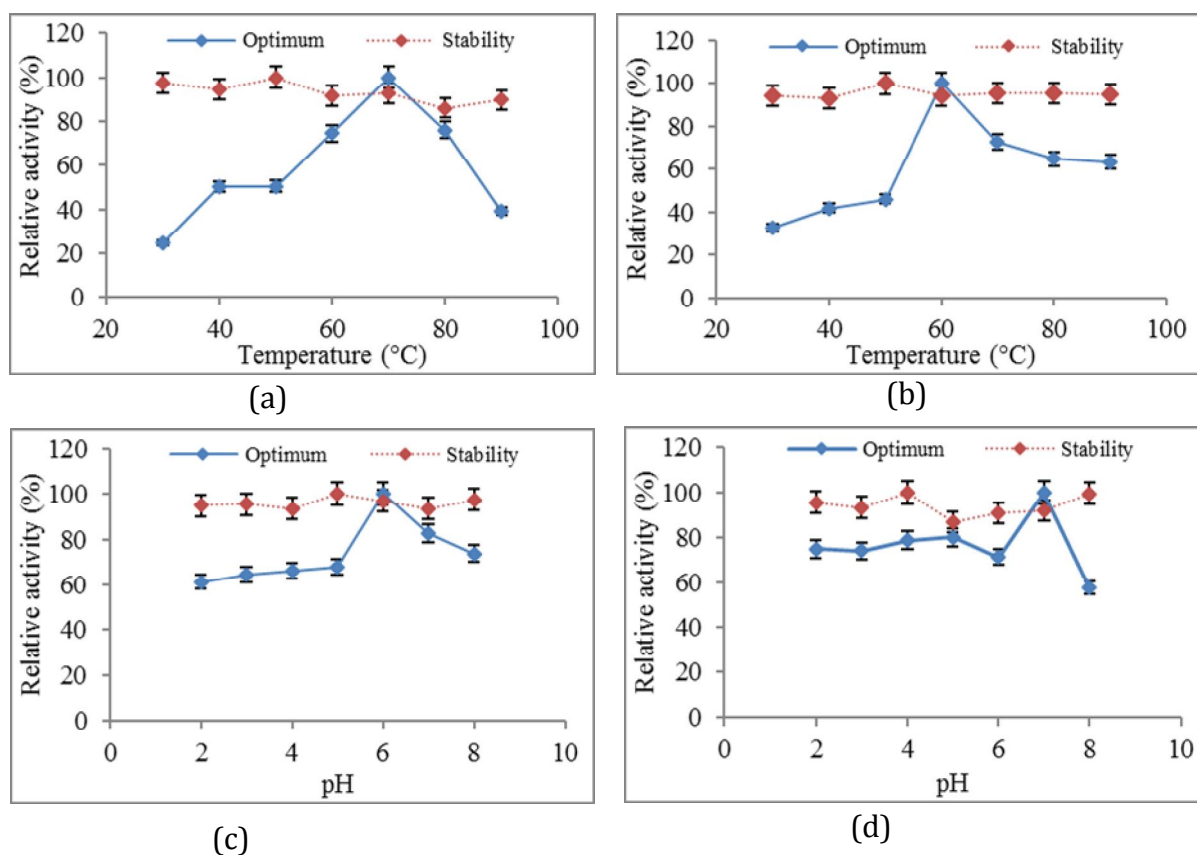


Fig.1 The effect of temperature and pH on mannanase activity from BLS.11-01 (a, c) and BLS.11-02 isolates (b, d).

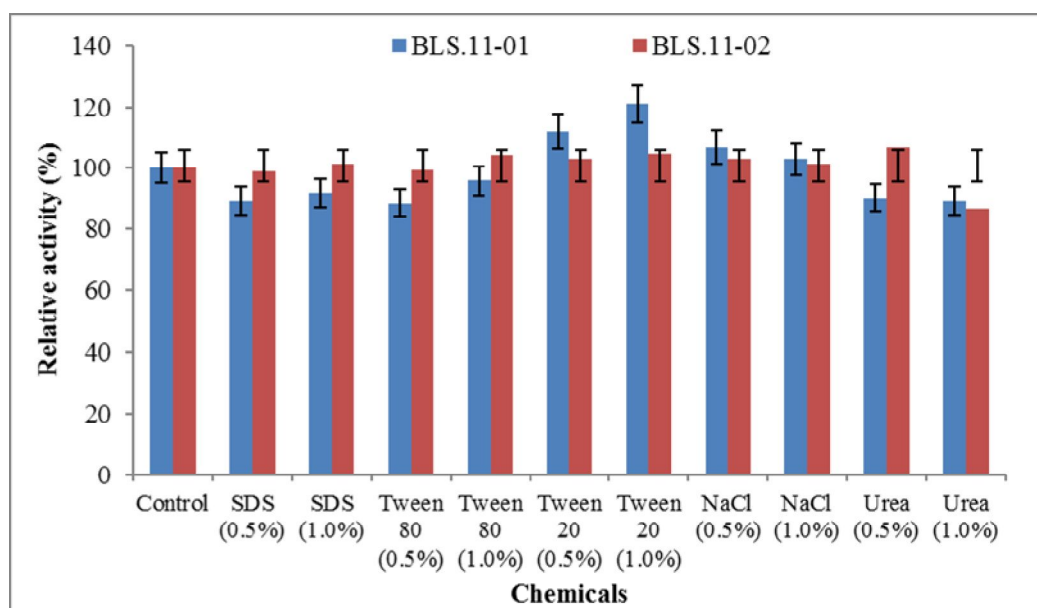


Fig. 2. Effect chemical reagents on the activity of the crude mannanase.

Effect of temperature on  $\beta$ -mannanase activity was performed by incubating the enzymes at various temperatures from 30°C-90°C.  $\beta$ -Mananase activity of BLS.11-01 and BLS.11-02 isolates had the optimum temperature at 70°C and 60°C. In general, Chauhan et al. [10] and Dhawan et al. [14] reported that the optimum activity of mannanase bacteria and fungi were in the range of 40°C-50°C. BLS.11-01 and BLS.11-02 isolates showed stability at wider temperature range of 30°C-

90°C. Mannanase activity of the isolate was relatively stable at 80% or more. Stability at high temperatures showed that BLS.11-01 and BLS.11-02 isolates had thermophilic properties. To determine that the properties of mannanase would be wider than both of these isolates, the characterization of the enzyme in subsequent purification step is needed to be done, thus the applications in various sectors can be maximized.

### 3.3 Effects of metal ions

Effect of metal ions on the mannanase activity of BLS.11-01 and BLS.11-02 isolates have been studied and is presented in Table 1. The activity of BLS.11-01 and BLS.11-02 were strongly influenced by the presence of  $\text{Co}^{2+}$  ions which could increase the relative activity of mannanase up to 9 and 12%.  $\text{Fe}^{2+}$  ions decrease the activity of mannanase up to 50%. While  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Ni}^{2+}$  ions only decrease the activity of mannanase up to 20-30%. The property of  $\text{Co}^{2+}$  ions as activators have been reported by Yoo et al. [52] who reported that increasing of activity of mannanase from *S. tendae* was up to 63%. Yang et al. [51] reported that the  $\text{Co}^{2+}$  ions enhanced the activity of mannanase from *Neosartorya fischeri* up to 10-13%.  $\text{Co}^{2+}$  ions might be associated with active A.Pase, which was essential for activity and for maintaining the conformational stability of the enzyme [43]. The effect of  $\text{Fe}^{2+}$  ions were not effective for mannanase activity from *S. sp. S27, Cellulosimikrobium sp. Strain HY.13* and *Bacillus subtilis WY34* [52, 42, 24, 22]. Microbial mannanases were affected by different metal ions and ion concentrations [20]. The ion effected on the enzyme activity might be related to the electric charge on enzyme surface. Furthermore, some enzymes required high concentrations of salts to maintain their high activity and stability [53].

### 3.4 Effect of detergents and denaturants

Effect of detergents and denaturants on mannanase activity of BLS.11-01 and BLS.11-02 is shown in Fig. 2. In case of detergents, tween 80 and SDS were detergents that could reduce the activity of mannanase in both isolates. While tween 20 could increase the mannanase activity of BLS.11-01 isolate significantly up to 20%. For BLS.11-02 isolate, tween 20 was less effective to increase the activity of mannanase. Detergent which was very effective at mannanase activity was beta-Mercaptoethanol which could increase the activity of mannanase from *Gloeophyllum trabeum* CBS900.73 up to 85% [49]. The same result have been reported by Hsiao et al. [20] and Johnvesly and Naik [23] who reported that  $\beta$ -mercaptoethanol could increase the activity mannanase of *Xanthomonas campestris* and *Bacillus sp. JB-99*. The possible reason was that  $\beta$ -mercaptoethanol might neutralize the oxidation effects of the S-S linkage between cysteine residues [51]. NaCl was denaturant which less effective, because it did not significantly affect the activity of mannanase. While Urea ass a denaturant which inhibit the activity of both mannanase isolates.

### 4. Conclusions

BLS.11-01 and BLS.11-02 were isolated from waste of sago hump. The potency of enzymes in hydrolysis of hemicellulose mannan has been evaluated. Enzymes were produced by these two isolates with high potency for their application because it has optimum activity at relative high temperatures and stable in wide temperature range, although it did not exhibit high activity at extreme pH. BLS.11-01 and BLS.11-02 activity was highly influenced by  $\text{Co}^{2+}$ , but inhibited by  $\text{Fe}^{2+}$ ,  $\text{Ni}^{2+}$ ,

$\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Ca}^{2+}$ . Chemical reagents did not affect on the activity of mannanase from both isolates significantly. The great potency was based on the availability of waste hemicellulose. The resulting enzyme of BLS.11-02 and BLS.11-01 isolates could be applied to produce manno oligosaccharides or other functional foods.

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