

# Enzymatic Hydrolysis of Copra Meal by Mannanase from *Streptomyces* sp. BF3.1 for The Production of Mannooligosaccharides

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Copra meal contained high polysaccharide mannan. Mannanase *Streptomyces* sp. BF3.1 efficiently hydrolyzed copra meal to mannoooligosaccharides. This research determined the optimum conditions of enzyme mannanase *Streptomyces* sp. BF3.1 to hydrolyze copra meal. The results of the hydrolysis products were analyzed concentrations of reducing sugars, total sugars and the degree of polymerization. In order to determine the type of product, mannoooligosaccharides were analyzed by thin layer chromatography and high performance liquid chromatography. The mannanase had an optimum condition at 70 °C and pH 6. Optimum conditions of hydrolysis was 10% copra meal concentration with incubation time of 5 h at 30 °C which able to produce a variety of mannoooligosaccharides products. Under such conditions, the yield of reducing sugar was 3.83 mg/mL with polymerization degree of 4. Analysis of mannoooligosaccharides by thin layer chromatography and high performance liquid chromatography revealed mannobiose, mannotriose, mannotetrose, mannopentose, and mannoheksose.

Keywords: copra meal, mannanase, mannoooligosaccharides, *Streptomyces* sp.

## INTRODUCTION

Indonesia is the world's second largest producer of copra. Indonesian copra meal production were predicted to be increased 520.000 tons (3.2%) from 1.56 to 1.58 million tons in 2013/2014 (USDA 2013). Copra meal is coconut residual cake that discharges as a byproduct in the process of oil extraction which is abundantly available and has a quite competitive price (Sundu & Dingle 2003). Copra meal has considerable potential as a source of protein and carbohydrates, but cannot be fully utilized as feed ingredients for monogastric animals (Mendoza *et al.* 1994). Limited use of copra meal is due to high level of non-starch polysaccharides (Purwadaria *et al.* 1995). Therefore, it is important to manage copra meal as prebiotic for animal feed formulas.

Mannan degradation by mannanase produced mannoooligosaccharides which serves as a functional food component to be used as a prebiotic (Yopi *et al.* 2006). The results of the analysis of industrial

products copra meal found that 60-70% of the carbohydrate content consists of  $\beta$ -mannan. Copra meal contains 61% mannan (Moreira & Filho 2008), 40-50% galactomannan, and the ratio of galactose:mannan = 1:14 (Regalado *et al.* 2000), which was discarded as a byproduct. Park (2008) reported that the hydrolysis of copra (brown copra) by mannanase is easier and economical for preparation of mannoooligosaccharides.

Mannoooligosaccharides prebiotics (MOS) are non-digested foodstuffs oligosaccharides which highly beneficial in influencing the intestinal microbiota by selectively stimulating the growth and activation of one or a number of bacteria in the gut (Gibson *et al.* 2004). Prebiotics are fermented in the colon by endogenous bacteria to release energy, metabolic substrates, lactic and short-chain carboxylic acids as end products of fermentation (Quigley 2010). Probiotics are living microorganisms which when administered in adequate amounts of colonies as commensal of the host conferring a health benefit on the host. The study of dietary mannoooligosaccharides on chicken caecal microflora by Fernandez *et al.* (2002) concluded

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that supplementation of feed with 2.5% MOS would have a significant influence to increase amount of *Bifidobacterium* spp. and *Lactobacillus* spp. and the decrease amount of group Enterobacteriaceae in the chicken intestines. Mannan oligosaccharides able to suppress the growth of Salmonella strains expressing fimbriae type-1 in the ceca of broiler chicks (Spring *et al.* 2000) and MOS could increase the amount of fat removed and reduces blood pressure in mammals (Kumao *et al.* 2006).

Microbial mannanases have become biotechnologically important since they target the hydrolysis of complex polysaccharides of plant tissues into simple molecules like manno oligosaccharides and mannoses (Dhawan & Kaur 2007). The diversity and production of mannanases by various species of the Actinomycetes from the Streptomycetes group i.e. *Streptomyces scabies* CECT 3340, *Streptomyces ipomoea* CECT 3341 (Montiel *et al.* 1999), *Streptomyces galbus* (Kansoh & Nagieb 2004), and *Streptomyces lividans* (Arcand *et al.* 1993). Besides these, *Rhodothermus marinus* a marine bacterium is a thermostable mannanase producing bacterium (Politz *et al.* 2000), Actinobacteria group, i.e. *Cellulomonas fimi* (Stoll *et al.* 1999) and *Thermomonospora fusca* KW3 (Hilge *et al.* 1998), fungi group i.e. *Trichoderma reesei* (Margolles *et al.* 1997), *Aspergillus niger* NCH-189 (Lin & Chen 2004) were described as mannan degraders with an ability to act on a wide variety of mannan substrates.

Actinomycetes have genetic and biochemical diversity, therefore it would be necessary to identify its potential to produce mannanase that can be used to produce prebiotic manno oligosaccharides from copra meal. This study determined the optimum conditions of mannanase *Streptomyces* sp. BF3.1 to hydrolyze copra meal and analyze manno oligosaccharides products by thin layer chromatography and high performance liquid chromatography.

## MATERIALS AND METHODS

### Copra Meal and Physico-Chemical Analysis.

The coconut residual cakes, usually called copra meal, were collected from Purwokerto, Jawa Tengah. Copra meal as a carbon source for medium formulation. Copra meal was milled and dried by sun drying for 12 h. The particle size of copra meal was 100 mesh (Figure 1). Copra meal physico-chemical properties including: moisture, protein, lipid, crude fibre and ash contents of the isolated samples were determined using approved methods (AOAC 1995).

**Microorganism.** Mannanase production was carried out using *Streptomyces* sp. BF3.1 from

actinomycetes collection of the Microbiology Laboratory, Departement of Biology IPB. Isolates was isolated from Taman Nasional Bukit Dua Belas, Jambi.

**Crude Enzyme Production.** Isolates was cultivated in liquid medium containing 0.5% copra meal, 0.075% peptone, 0.14%  $(\text{NH}_4)_2\text{SO}_4$ , 0.2%  $\text{KH}_2\text{PO}_4$ , 0.03%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03%  $\text{CO}(\text{NH}_4)_2$ , 0.03%  $\text{CaCl}_2$ , 0.0005%  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.00016%  $\text{MnCl}_2 \cdot 7\text{H}_2\text{O}$ , 0.00014%  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.0002%  $\text{CoCl}_2$ . Media were sterilized at 121 °C for 15 min. Fermentation was performed for 6 days at 2.24 xg, 30 °C (Taitec). The crude mannanase enzyme preparation was obtained as the culture supernatant by centrifugation (8050 xg, 10 min, 4 °C).

**Crude Enzyme Mannanase Assay.** The mannanase activity was assayed according to Meryandini *et al.* (2008) by incubating 0.5 mL of the enzyme solution with 0.5 mL of locus bean gum solution (0.5% w/v) (Merck, Darmstadt, Germany) prepared in phosphate buffer pH 6 (50 mM) at 30 °C for 30 min. The reaction was stopped by immersing the test tube in boiling water for 20 min. Absorbance was measured in a spectrophotometer at  $\lambda = 540$  nm (Hitachi, U-3900H, Tokyo, Japan). The reducing sugars liberated were estimated by the DNS methods (Miller 1959). One unit was defined as the amount of enzyme that could produce 1  $\mu\text{mol}$  of reducing sugar (mannose base) for 1 min under the assay condition.

**Crude Enzyme Characterization.** The effect of pH on the crude mannanase activity was determined using 0.5% locus bean gum suspended in buffers consisting of citrate (pH 3-5), phosphate (pH 6-8) and glycine-NaOH (pH 9-10) at 50 mM. The effect of temperature on the crude mannanase activity was assayed at temperature values ranging from 30 to 100 °C.

### Enzymatic Hydrolysis of Copra Meal.

Enzymatic hydrolysis was carried out under various conditions, such as different substrate concentrations (w/v) 1, 5, and 10% in enzyme solution and the reaction time 1, 3, 5, and 24 h. Reactions were carried out in 50 mL Erlenmeyer flasks containing 10 mL of reaction mixtures in rotary shaker (Stuart orbital incubator S1500, Staffordshire, United Kingdom) at 30 °C. Samples were taken at regular intervals (after 1, 3, 5, and 24 h). Reactions were stopped in boiling water for 20 min.

**Analysis of Mannooligosaccharides.** Product hydrolysis was analyzed by calculating the reducing sugar, total sugar content, and degree of polymerization. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) were used for analysis of manno oligosaccharides.

Reducing sugars were determined by DNS method (Miller 1959). Analysis of the total sugar content was performed by applying the phenol-sulfuric acid method with modifications described by Dubois *et al.* (1956). The degree of polymerization was calculated according to the proportion of the total sugar content and reducing sugar. TLC of manno oligosaccharides products was carried out on silica gel 60F<sub>254</sub> plates (Merck Art 20 x 20 cm, Darmstadt, Germany). All samples were applied in equal quantities (3  $\mu$ L) and then resolved with a solvent mixture of n-butanol:acetic acid:water (12:6:6 volume). Spot were visualized by spraying the sugar color (0.2 g diphenylamine, 0.2 mL aniline, 10 mL acetone, 1.5 mL phosphate acid) and subsequently heating at 120 °C for 10 min. Manno oligosaccharides products were analyzed by HPLC under the following conditions: column 250 x 4.6 mm i.d. Zorbax SIL coated with 3-amino propyl silane, mobile phase was acetonitrile and distilled water in ratio 75:25 (v/v), column temperature 30 °C, flow rate 1.4 mL/min and detector Agilent Technologies 1200 series refractive index monitor (RID).

## RESULTS

**Production dan Characterization of Crude Mannanase.** Copra meal contains crude fibers carbon sources and 20.02% protein (Table 1) sufficiently high as a source of nitrogen for growth media. The optimum incubation time for *Streptomyces* sp. BF3.1 to produce the highest enzyme activity (0.98 U/mL) was 120 h (Figure 2).

The effects of pH and temperature on crude mannanase were investigated as shown in Figure 3 and 4. Mannanase enzyme optimal pH was in the range of 6-8, with the highest activity of 0.74 U/mL



Figure 1. Copra meal.

Table 1. Nutrient composition (% dry matter) of copra meal

Moisture	Protein	Fat	Crude fibre	Ash
13.33 $\pm$ 0.13	20.02 $\pm$ 0.54	0.78 $\pm$ 0.32	7.62 $\pm$ 0.42	8.51 $\pm$ 0.01

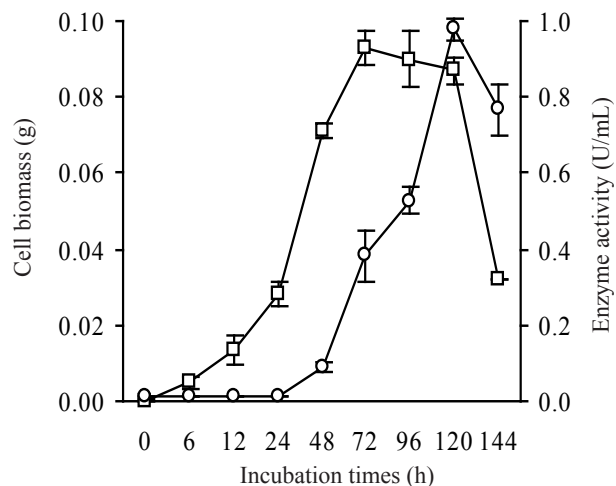


Figure 2. Measurement of cell biomass and enzyme activity of crude mannanase *Streptomyces* sp. BF3.1 grown on 0.5% copra meal medium, pH 6, temperature 30 °C, agitation 200 rpm.  $\square$ : Cell biomass and  $\circ$ : crude mannanase activity.

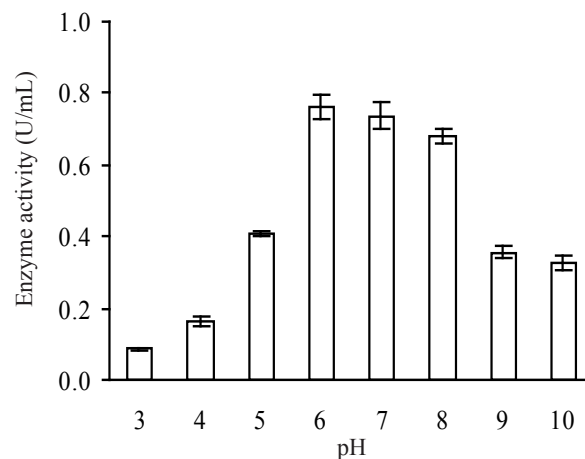


Figure 3. Characterization of crude mannanase *Streptomyces* sp. BF3.1 activity in various pH at 30 °C.

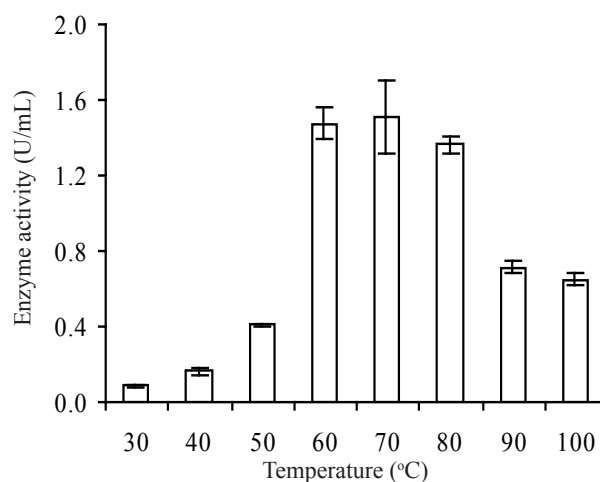


Figure 4. Characterization of crude mannanase of *Streptomyces* sp. BF3.1 activity in various temperature at buffer phosfat pH 6.

at pH 6. The optimum temperature was 70 °C with activity of 1.50 U/mL. The stability of the enzyme at 4, 30, and 70 °C was observed. Mannanase remained stable for 24 h at 4 and 30 °C, whereas enzyme activity decreased drastically at 70 °C and lost its activity after 2 h (Figure 5).

**Enzymatic Hydrolysis of Copra Meal.**

Enzymatic hydrolysis of copra meal conducted to three variations of the substrate concentration: 1, 5, and 10% (w/v), in 10 mL enzyme solution (18.7 U). Reducing sugar concentration increased at 1 to 5 h and declined rapidly to 24 h on all various substrate concentrations (Figure 6). Total sugar concentration

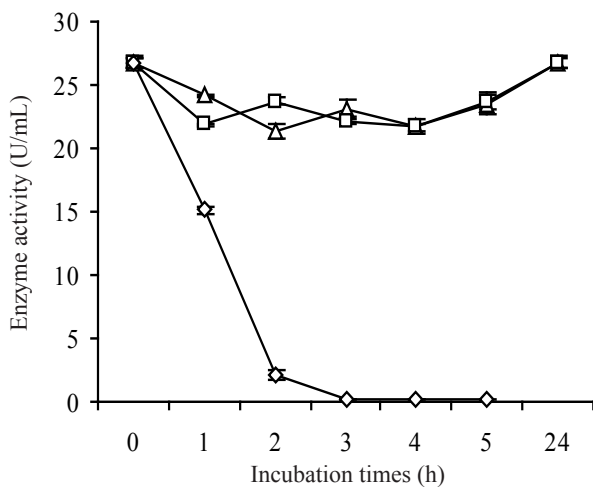


Figure 5. Characterization of crude mannanase of *Streptomyces* sp. BF3.1 activity in various incubation time at storage temperature 4 °C (▲), 30 °C (◻), and 70 °C (◊).

tend to be similar every hour of incubation (data not shown). Based on the concentration of total sugar and reducing sugar obtained, we can calculate the degree of polymerization. Value of the degree of polymerization of the hydrolysis products obtained was ranging from 2-7 (Table 2).

**Analysis of Mannooligosaccharides.** Results of thin layer chromatography (Figure 7) showed that the mannanase was able to hydrolyze mannan copra (1, 5, and 10%); with an incubation time of 5 h could

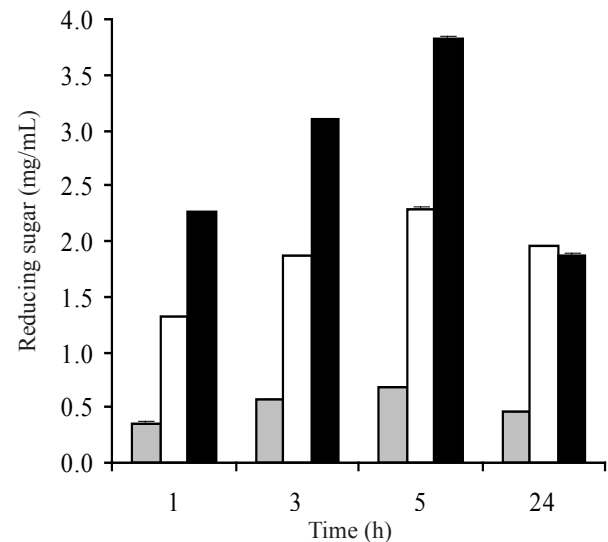


Figure 6. Analysis of reducing sugar content of copra meal hydrolyzed by mannanase enzyme of *Streptomyces* sp. BF3.1. The copra meal concentration were 1% (◻), 5% (◻), and 10% (◼) in 10 mL enzyme solution (1.87 U/mL) at 30 °C.

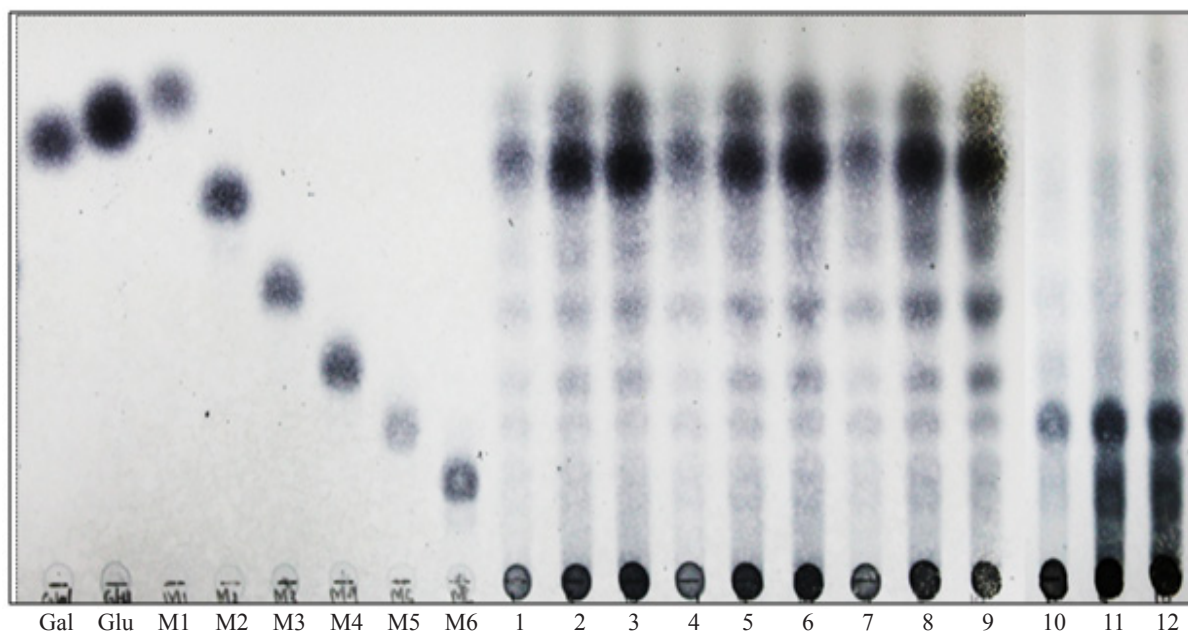


Figure 7. Thin Layer Chromatography analysis of copra meal (1, 5, 10%) hydrolyzed by mannanase *Streptomyces* sp. BF3.1. Standard Gal: Galactose; Glu: glucose; M1: mannose; M2: mannobiose; M3: mannotriose; M4: mannotetrose; M5: mannopentose; M6: mannoheksose. Sample Line 1, 1% 1 h; line 2, 5% 1 h; line 3, 10% 1 h; line 4, 1% 3 h; line 5, 5% 3 h; line 6, 10% 3 h; line 7, 1% 5 h; line 8, 5% 5 h; line 9, 10% 5 h; line 10, 1% 24 h; line 11, 5% 24 h; line 12, 10% 24 h.

produce a variety of manno oligosaccharides products including mannobiose, mannotriose, mannotetrose, mannopentose, and mannoheksose, except at 24 h it only produced mannopentose and mannoheksose. The optimum conditions of hydrolysis was 10% copra meal concentration at incubation time of 5 h. This condition was able to produce a variety of manno oligosaccharides products and reducing sugar of 3.83 mg/mL.

The results of the analysis of hydrolysis of 10% copra meal with incubation time for 5 h using HPLC showed seven peaks (Figure 8). Mannose and glucose monomers detected in the first and third peak with a retention time of 4.176 and 5.478 min. Manno oligosaccharides products detected were mannobiose (5.051 min), mannotriose (6.347 min), mannotetrose (7.438 min), mannopentose (8.695 min), and mannoheksosa (10.609 min).

Table 2. Degree of polymerization (DP) analysis with variations of copra meal concentration were 1, 5, and 10% in 10 mL enzyme solution (1.87 U/mL), 30 °C

Concentration of substrat (%)	Hydrolysis time (h)	DP
1	1	4
	3	3
	5	2
	24	2
5	1	4
	3	3
	5	3
	24	3
10	1	7
	3	5
	5	4
	24	7

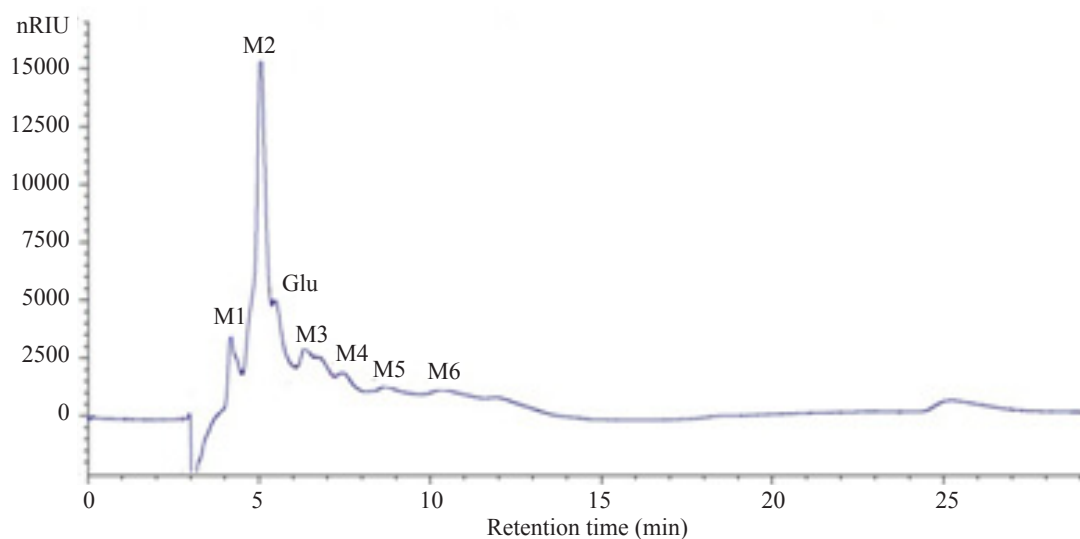


Figure 8. High performance liquid chromatography analysis of 10% copra meal with incubation time for 5 h hydrolyzed by mannanase *Streptomyces* sp. BF3.1. Chromatography conditions: column 250 x 4.6 mm i.d. Zorbax SIL coated with 3-amino propyl silane, eluent acetonitrile and water in ratio 75:25 (v/v), column temperature 30 °C, flow rate 1.4 mL/min and detector refractive index monitor (RID).

## DISCUSSION

Copra meal contains approximately 43-45% mannan polysaccharide of total carbohydrates (Saittagaroon *et al.* 1983) were used as the carbon source for medium formulas. The results of the study of Khuwijitjaru *et al.* (2012), copra meal containing  $\pm 79.77\%$  mannose,  $\pm 12.80\%$  glucose,  $\pm 6.12\%$  galactose, and  $\pm 1.31\%$  arabinose of the total carbohydrate 68.99%. Based on data, the copra meal hydrolyzed by mannanase would produce manno oligosaccharides.

Enzyme activity and time production of mannanase *Streptomyces* sp. BF 3.1 was different with other microbes. *Streptomyces* sp. BF3.1 to produce mannanase with activity 0.98 U/mL (120 h incubation time). *Streptomyces scabies* CECT 3340 and CECT 3341 *Streptomyces ipomoea* mannanase enzyme capable of producing 294.3 and 242.9 U/L, with an incubation time of 96 h (Montiel *et al.* 1999) and *Streptomyces* sp. PG-08-03 mannanase activity 15 U/mL for 72 h of incubation (Bhoria *et al.* 2009). *Aspergillus niger* NCH-189 (Lin & Chen 2004) and *Bacillus subtilis* strain NM-39 (Mendoza *et al.* 1994) fermented copra meal on media capable of producing the enzyme mannanase each at 2.6 U/mL (3 d incubation time) and 2.2 U/mL (24 h incubation time).

**Characterization of Mannanase *Streptomyces* sp. BF3.1.** Mannanase *Streptomyces* sp. BF 3.1 was optimum at pH 6 similar to mannanase *Aspergillus awamori* K4 (Kurakake & Komaki 2001). Mannanase *Streptomyces* sp. BF3.1 was optimum at 70 °C

similar to  $\beta$ -mannanase thermophilic actinomycetes *Thermomonospora fusca* optimum temperature of 80 °C (Hilge *et al.* 1998), *Aspergillus awamori* K4 mannanase activity was optimum at 80 °C (Kurakake & Komaki 2001) and *Brevibacillus bortstelensis* was optimum at 90 °C (Utami *et al.* 2013). Compared to previous research characterizing other activities of *Streptomyces* mannanase, i.e. *Streptomyces lividans* was optimum pH 6.8, 58 °C (Arcand *et al.* 1993), and *Streptomyces galbus* NR pH 6.5, 40 °C (Kansoh & Nagieb 2004), it can be concluded that almost all *Streptomyces* group has an optimum pH range of 6-7 and mannanase of *Streptomyces* sp. BF3.1 has a higher optimum temperature than the mannanase of *Streptomyces* from previous studies.

Stability of mannanase *Streptomyces* sp. BF3.1 was remain stable at 30 °C for 24 h and the enzyme lost its activity at 70 °C after 2 h, compared to previous study of mannanase activity *Cellulosimicrobium* sp. strain HY-13 which was remain stable at 37 °C for 60 min incubation, lost its activity after incubation at 60 °C for 10 min (Kim *et al.* 2011) and mannanase *Klebsiella oxytoca* 2-3 CW retains 82% of its activity when incubated at 60 °C for 30 min incubation (Titapoka *et al.* 2008). This indicates that the mannanase of *Streptomyces* sp. BF3.1 has better stability compared with the results of previous studies and effectively used to hydrolyze copra meal for 24 h at room temperature.

**Enzymatic Hydrolysis of Copra Meal and Analysis of Mannooligosaccharides.** Mannanase *Streptomyces* sp. BF3.1 18.7 U in 10 mL was used to hydrolyze copra meal at 30 °C for 24 h. Degree of polymerization (DP) of the hydrolysis products obtained was ranging from 2-7, similar to the results of Abe *et al.* (1994) where copra meal hydrolyzed by mannanase *Bacillus circulans* and *Bacillus albei* produce mannooligosaccharides DP 2-4. Konjac flour was hydrolyzed by mannanase *Bacillus* sp. MSJ-5 produces mannooligosaccharides DP 2-6 (Zhang *et al.* 2009). Mannanase *Brevibacillus bortstelensis* was hydrolyzed palm kernel cake (PKC) to produce mannooligosaccharides with DP 10 obtained after 72 h of incubation with ratio substrate 1.5% PKC (Utami *et al.* 2013). Hydrolysis of guar and locust bean gums with  $\beta$ -mannanases from *Penicillium oxalicum* resulted in the production of low molecular-weight oligomers, which accounted for 92% of the total released saccharides. The DP range of oligomers was 2-7 for guar gum and 2-6 for locust bean gum (Kurakake *et al.* 2006). Oligosaccharides have a degree of polymerization between monomer

and polysaccharide, DP 3-10 (Weijers *et al.* 2008). Thin layer chromatography can reveal the degree of polymerization of oligosaccharides (Patel & Goyal 2011).

Copra meal contains mannobiose as detected by TLC (data not shown). Mannobiose concentration increased when oligosaccharides hydrolyzed randomly by mannanase. Hydrolysis by  $\beta$ -mannanases usually produce mannobiose and mannotriose products (Adenmark *et al.* 1998). Hydrolysis of copra meal by mannanase BF 3.1 yielded mannose, glucose and unknown compounds which moved further than mannotriose and mannotetraose on TLC. Hydrolysis of mannan resulted in various kinds of unknown oligosaccharides and unknown substances. These unknown compounds may be mannooligosaccharides carrying side chains that might be resulted from transferase activity of the hydrolase.

Mannooligosaccharides products in this study was similar with results of copra mannan hydrolysis by mannanase *Bacillus* (Abe *et al.* 1994; Hossain *et al.* 1996) and hydrolysis galactomannan by thermostable mannanase *Paenibacillus illinoisensis* ZY-08 (Lee *et al.* 2010). Coffee mannan hydrolysis by mannanase *Sclerotium rolfsii* also generated mannooligosaccharides including mannobiose, mannotriose and mannotetrose (Sachslehner *et al.* 2000).  $\beta$ -mannanases attacked internal glycosidic bonds of the mannan backbone chain, releasing short  $\beta$ -1,4-mannooligosaccharides (Van Zyl *et al.* 2010). The presence of mannose and glucose (monosaccharide) in the hydrolyzed mixture is due to the activity  $\beta$ -glucosidases and  $\beta$ -mannosidase.  $\beta$ -glucosidases remove the 1,4-glucopyranose units at the non-reducing end of the oligomers galactoglucomannan of copra meal (Moreira & Filho 2008).  $\beta$ -mannosidase, an exo-type enzyme, cleaves  $\beta$ -1,4-linked mannosides, releasing mannose from the non reducing end of mannans and mannooligosaccharides (Dhawan & Kaur 2007).

In conclusion, we have mannanase *Streptomyces* sp. BF3.1 which could hydrolyzed copra meal to mannooligosaccharides. The optimum conditions of hydrolysis is 10% copra meal concentration at 30 °C for 5 h incubation time. This condition was able to produce a variety of products mannooligosaccharides. Further study will be necessary to separate the products of monomers and oligosaccharides using high performance liquid chromatography to obtain pure mannooligosaccharides to be tested on probiotics and required the structural analysis of mannooligosaccharides.

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