

Isolation and characterization of mannanase, xylanase, and cellulase from marine bacteria *Bacillus* sp.

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Abstract. *Yopi, Djohan AC, Rahmani N, Jannah AM. 2017. Isolation and characterization of mannanase, xylanase, and cellulase from marine bacteria Bacillus sp. Biofarmasi J Nat Prod Biochem 15: 15-20.* Isolation, identification, and characterization of mannanase, xylanase, and cellulase-producing indigenous marine bacteria have been conducted from a total of 20 isolates. Based on 16S rDNA sequence analysis, three potential isolates are obtained and identified *Bacillus subtilis* (M8), *Bacillus tequilensis* (X4), and *Bacillus cereus* (C9). The potential strains M8, X4 and C9 can produce mannanase, xylanase and cellulase activities such as 9.5 U/mL; 0.36U/mL;0.56U/mL with optimum pH and temperature 6.0;50°C, 5.5;70°C and 8;50°C, respectively. Based on the TLC analysis, mannanase from M8 and xylanase from X4 has the potential to hydrolyze mannan and xylan for producing oligosaccharides with the size around tri-hexasaccharide as the main product.

Keywords: *Bacillus* sp., cellulase, hemicellulase, marine bacteria, oligosaccharide.

INTRODUCTION

The utilization of cellulose by microorganisms involves a substantial set of fundamental phenomena beyond those associated with enzymatic hydrolysis of cellulose (Lynd et al. 2002). Marine biodiversity microorganism has high potency for biotechnology and the enzymatic environment in Indonesia. There is a deep interest in searching for new enzymes source of cellulase and hemicellulase for industrial application (Sukumaran et al. 2005).

Marine microorganisms have been regarded as a reservoir, not only for novel natural products but also for valuable genes and enzymes (Guo et al. 2013). Several xylanases produced by marine microorganisms from special habitats, such as the deep-sea hydrothermal field (Wu et al. 2006), the Antarctic marine soil (Collins et al. 2002), and marine sediment (Guo et al. 2009), were shown to have special properties, such as hyperthermostability (Wu et al. 2006), cold adaptation (Collins et al. 2002; Guo et al. 2009) and salt-tolerance (Guo et al. 2009; Liu et al. 2012).

The utilization of marine bacteria to produce cellulase and hemicellulase has not been used widely in Indonesia. There is a report related to isolated marine bacteria from Pari island, which have the ability to produce mannanase (Djohan 2014). We have already collected marine microbes from Bali Island and already identified them using 16S rRNA as cellulase and hemicellulase marine microbe belonging to *Bacillus* sp.

This research intends to isolate and characterize cellulase and hemicellulase deriving from marine microbes for new enzyme applications in biotechnology fields, especially to convert abundant cellulose and hemicellulose biomass into monosaccharides that can be converted into

bioethanol through fermentation yeast.

MATERIALS AND METHODS

Isolation mannanase, xylanase, and cellulase producing marine bacteria

Mannanase, xylanase, and cellulase-producing bacteria were isolated from seawaters and sediment collected on Bali Island. The Locust Bean Gum (LBG), CMC, and Xylan are mediums for screening and purifying bacteria and contain 0.5% of substrate (LBG, CMC, and Xylan), 0.075% of peptone, 0.05% of extracted yeast which were then diluted in Artificial Sea Water (ASW) at pH 6.0 (Mandels and Sternberg 1976). The purified colonies were preserved at 4°C for further study.

Screening of mannanase, xylanase, and cellulase producing marine bacteria

Pure cultures of bacteria isolates were transferred individually in CMC, xylan, and LBG agar plates. After incubation for one day, these agar plates were flooded with 0.2% red congo and allowed to stand for 30 min at room temperature for counterstaining the plates, NaCl 1 M solution was thoroughly used. The bacteria colonies with the largest clear zone were selected to identify and produce cellulase, xylanase, and mannanase in submerged fermentation.

Identification and phylogenetic analysis 16S rDNA sequence

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) technique using a pair of primer 9F: 5'-AGRGTTTGATCMTGGCTCAG-3' and 1492R:

5'TACGGYTACCTTGTTAYGACTT-3' (Burggraft et al. 1992). The obtained bands were stained in Bio and were visualized by a UV transilluminator.

Production of mannanase, xylanase, and cellulase

For fermentation, Mannanase, xylanase, and cellulase were produced by three potential isolates (M8, X4, and C9), precultured in a 100 mL flask containing 10 mL of ASW cultivated at 30°C for one day. The preculture medium was seeded into a 300 mL flask containing 30 mL of ASW medium with adjusted cell 0.02 (OD $\lambda=660$) and incubated at 30°C for six days.

Mannanase, xylanase, and cellulase activity assay

Mannanase, xylanase, and cellulase activity were assayed by measuring the reducing sugars using the dinitro salicylic acid (DNS) method (Miller 1959). Cellulase activity was measured using substrate 0.5% of CMC (Sigma) and dissolved in 50 mM sodium phosphate buffer, pH 7.0. An appropriately diluted enzyme solution (250 μ L) was incubated with 250 μ L of the substrate solution at 60°C for 15 min. The amount of reducing sugars released in the enzyme reaction was assayed by mixing 750 μ L of DNS solution, heating it at 100°C for 10 min, then cooling it on ice for 10 min, and measuring the absorbance at 540 nm. One unit of cellulase activity is defined as the amount of enzyme that releases 1 μ mol of D-glucose per minute under the experimental condition given.

Xylanase activity was measured using 250 μ L of the substrate solution of 0.5% xylan (Sigma). An appropriately diluted enzyme solution (250 μ L) dissolved in 50 mM sodium phosphate buffer with pH 7.0 was incubated at 60°C for 15 min. After incubation, 750 μ L DNS solution was added to end the reaction. The amount of reducing sugars released in the mixture was determined, with xylose as the standard using the DNS method (Miller 1959). One unit of xylanase activity is defined as the amount of enzyme capability to release 1 μ mol of xylose per minute under the assay condition.

Mannanase activity was measured using a substrate of 0.5% LBG (sigma) which was dissolved in 50 mM of sodium phosphate buffer, pH 7.0. An appropriately diluted enzyme solution (250 μ L) was incubated with 250 μ L of the substrate solution at 60°C for 15 min. The amount of reducing sugars released in the enzyme reaction was assayed by mixing 750 μ L of DNS solution, heating it at 100°C for 10 min, cooling it on ice for 10 min, and measuring the absorbance at 540 nm. One unit of mannanase activity is defined as the amount of enzyme that liberates 1 μ mol of D-mannose per minute under the experimental condition given.

Characterization of optimum pH and temperature

The optimum enzyme activity pH was examined at pH 3.0-10.0 under standard assay conditions. Fifty mM of various buffers were used: sodium citrate (pH 3.0-5.0), sodium phosphate (pH 6.0-8.0), and glycine NaOH (pH 9.0-10.0). The enzyme reaction was incubated at 50°C for 15 min in the presences of 0.5% (w/v) LBG for mannanase, 0.5% (w/v) xylan for xylanase and 0.5% (w/v) CMC for

cellulose. The effect of temperature on enzyme activity was performed at a temperature ranging from 30-90°C in 50 mM acetate buffer at optimum pH for 15 min.

Cellulosa and hemicellulose hydrolysis analysis by using thin-layer chromatography

Hydrolysis on mannan substrates (LBG, Konjac glucomannan, Ivory nut, porang), xylan (xylan), and cellulose (CMC) was carried out at 50°C in 50 mM of sodium phosphate buffer, pH 6, containing 0.5% for each substrate. The enzyme-substrate ratio (v/v) was 1: 1 and the reaction time was from 0, 0.5, 1, 2, 4, 8, 12 and 24 hours. The reaction was carried out in 1.5 mL of Eppendorf containing 100 μ L of reaction mixtures in the dry block. Thin Layer chromatography (TLC) of oligosaccharides was carried out on silica gel 60F₂₅₄ plates (Merck Art 20x20 cm) and eluent with a solvent mixture of n-Butanol/Acetic acid/Water (12: 6: 6, v/v/v). The spots were visualized by TLC dye reagent, i.e., DAP (0.5 g α -diphenylamine, 25 mL acetone, 2.5 mL phosphate acid, 0.5 ml aniline) and subsequently heated at 120°C for 15 min. The samples were applied in equal quantities (4 μ L). Glucose, mannose (Sigma-Aldrich, U.S.A.), mannoiose (M2), mannotriose (M3), mannotetraose (M4), and mannopentaose (M5) and mannohexaose (all from Megazyme, Ireland) were used as a standard.

RESULTS AND DISCUSSION

Isolation of cellulose and hemicellulose from marine bacteria

The research collected 20 isolates from a screening medium of artificial seawater and bacteria that could produce several types of enzymes, such as mannanase, xylanase, and cellulase enzyme. Then they were screened further for their abilities to excrete enzymes in some substrates, such as the locust bean galactomannan substrate, which is a medium for getting the mannanase, xylene for getting the xylanase, and CMC for getting the cellulase potential marine microbe. Isolation on cellulose and hemicellulose of marine bacteria using some substrate (locust bean gum, xylan, CMC) has been done using congo red analysis (Figure 1). For the mannolytic microbe, we obtain 11 isolates that produce mannanase. Still, only the M8 isolate produces the highest mannanase activity; for xylanase, we obtain one isolate X4, and for cellulase, we obtain one isolate C9.

Identification of the purified isolates based on the 16S rDNA sequence

Based on the sequence analysis of the 16S rDNA gene against the GenBank database, there is an indication that 3 selected isolates were closely related to the members of the genus *Bacillus*. The phylogenetic analysis showed about 99% similarity between isolate M8 and *Bacillus subtilis*, isolate X4 and *Bacillus tequilensis*, and isolate C9 and *Bacillus cereus* (Table 1).

Production of mannanase, xylanase, and cellulase

The crude enzyme production was done from marine bacteria (M8 isolates for mannanase, X4 for xylanase, and C9 for cellulase) for 144 hours of culture (Figure 2), which were grown in ASW medium with shaking (180 rpm) at 30°C.

The members of the genus *Bacillus* produce large varieties of extracellular cellulase and hemicellulase, such as cellulase, mannanase, and xylanase, which are significantly important enzymes for industrial demand. These quantitative enzyme tests have been conducted to determine the ability of each isolate to produce an enzyme using commercial substrates as a carbon source. The isolates produce enzymes using commercial substrate as a carbon source with maximal activity at 72 hours of culture, such as xylanase 0.36 U/mL, mannanase 9.5 U/mL, and cellulase 0.56 U/mL.

Characterization of optimum pH and temperature

The effect of pH on the activity of Mannanase M8, xylanase X4, and cellulase C9 was carried out in the range 3-10, as shown in Figure 3. The crude mannanase M8 was active at a wide pH range from 5.0-10.0, xylanase X4 from 3.0 to 10.0, and cellulase C9 from 3.0-9.0. The highest mannanase, xylanase, and cellulase activity were observed at pH 6.0, 5.5, and 8.0, respectively (Figure 3.A, 3.C, 3.E).

The increase or decrease of pH depends on enzyme activity. The effect of temperature was studied on each enzyme activity by varying the value from 30°C to 100°C. The effects of temperature on the activity of Mannanase M8 were shown in Figure 5.B with the optimum temperature at 70°C and high enzyme activity of 9.5 U/mL. The report of xylanase X4 was shown in Figure 5.D, in which the optimum temperature is 70°C, with the activity of 2.2 U/mL. The report of cellulase C9 is shown in Figure 5.F, which optimum temperature is 50°C with an activity of 0.26 U/mL. The enzyme activity increased when the temperature increased from 40°C, but the activity started to decline as the temperature increased above 80°C and became completely denatured at 100°C. Similar findings were also reported by some species, i.e., *Bacillus* sp and *B.subtilis* YJ1, which have an optimum temperature of 50°C (Kim et al. 2011).

Table 1. The phylogenetic analysis showed about 99% similarity between the isolated M8 and *Bacillus subtilis*, isolate X4 and *Bacillus tequilensis*, and isolate C9 and *Bacillus cereus*

Code of strain	Isolated from	Identified as*
M8	Sea water	<i>Bacillus subtilis</i>
X4	Sea water	<i>Bacillus tequilensis</i>
C9	Sea water	<i>Bacillus cereus</i>

Note: *by 16S rRNA gene sequencing

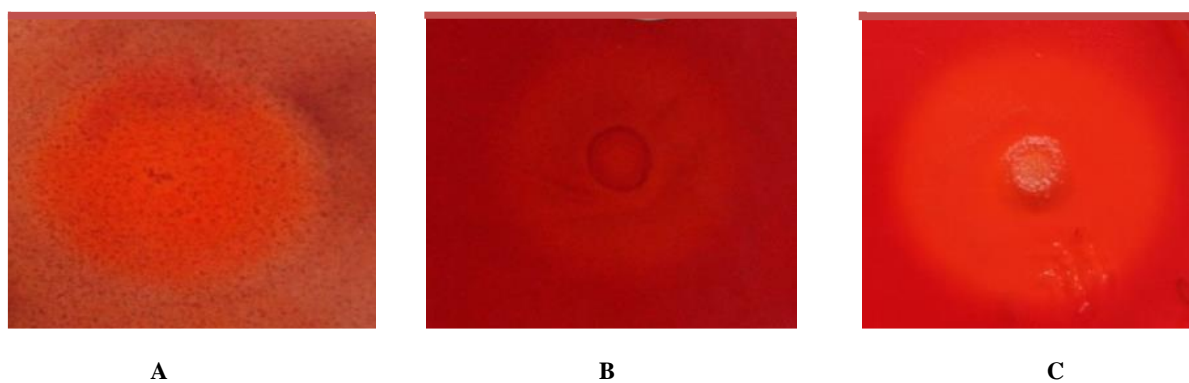


Figure 1. Congo red analysis from (A) mannolytic M8, (B) cellulolytic C9, and (C) xylanolytic X4 in a medium that uses a specific substrate (LBG, xylan, and CMC) for 24 hours of incubation

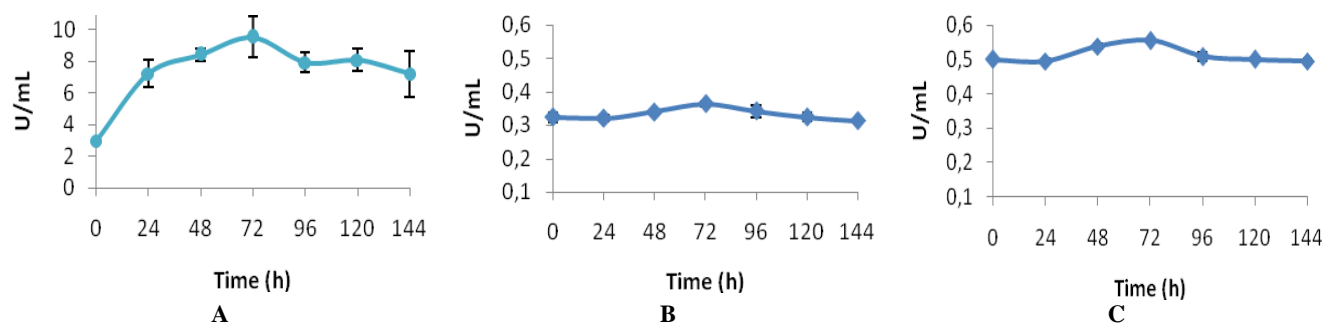


Figure 2. Crude enzyme production from potential marine bacteria with 24 hours retention times for initial optimization, such as mannanase M8 (A), xylanase X4 (B), and cellulase C9 (C).

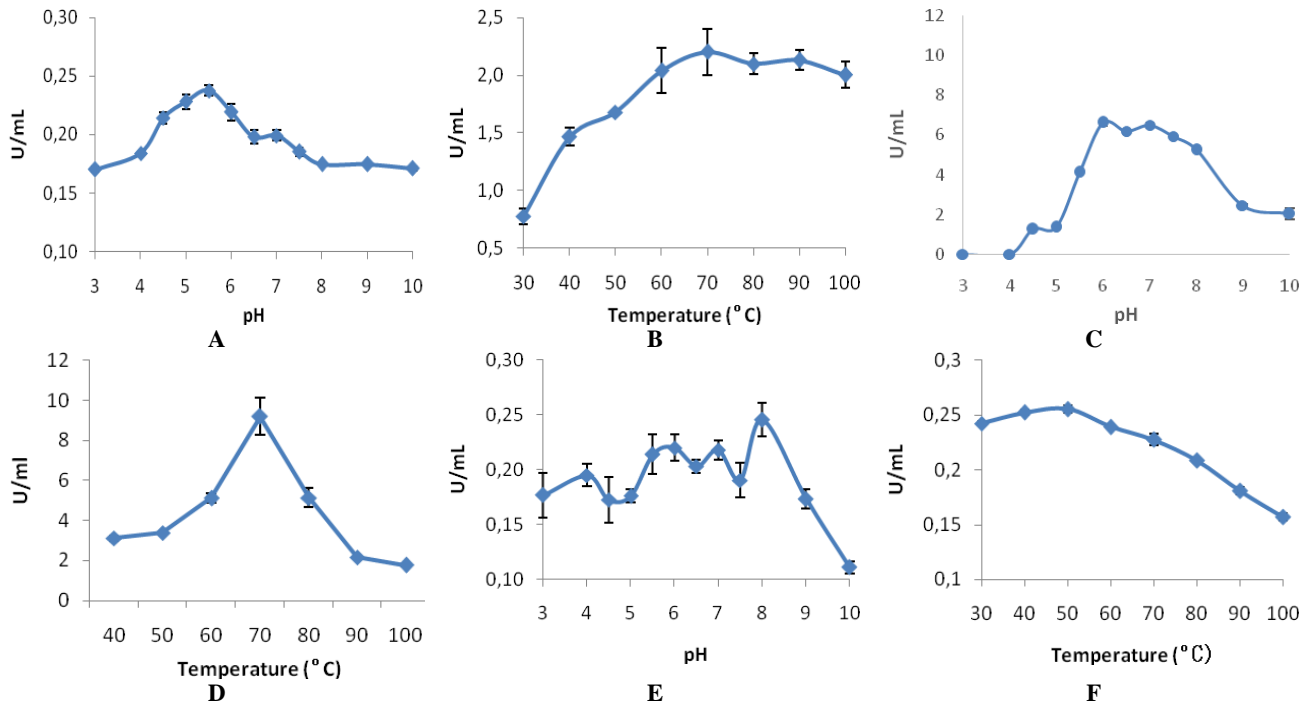


Figure 3. Characterization of pH and temperature xylanase from isolate X4 (A, B), mannanase from isolate M8 (C, D), and cellulase from isolate C9 (E, F)

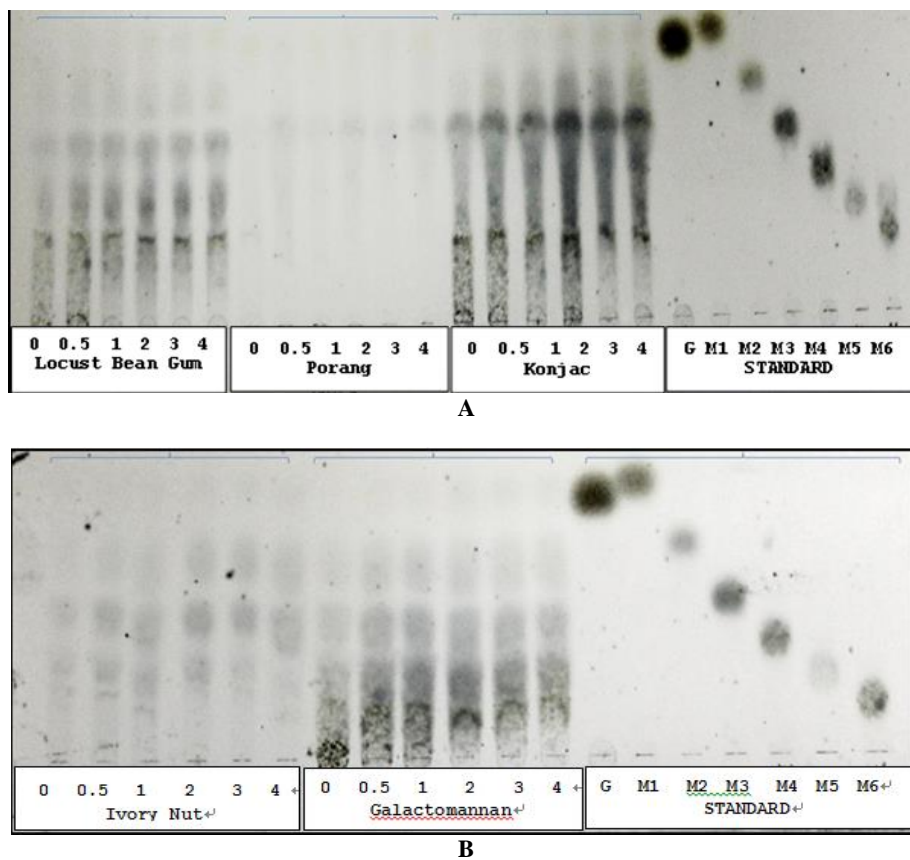


Figure 4. Thin-layer chromatography analysis of hydrolysis products mannanase M8 using various mannans as substrates, namely, LBG, porang, konjac glucomannan (A) and galactomannan, and ivory nut (B). Standards (STD): glucose (G), mannobiose (M2), mannotriose (M3), mannotetraose (M4), mannopentose (M5), and mannohexaose (M6). Substrate: enzyme (v/v) = 1: 10 and 1: 5, Substrate concentration 0.5% in 50 mM sodium phosphate buffer (pH 6.0), reaction time (hours): 0, 0.5, 1, 2, 3 and 4 at 30°C condition.

Product analysis by thin-layer chromatography

The hydrolysis of mannan sources such as locust bean gum, porang, konjac, ivory nut, and galactomannan by marine bacteria M8 was shown in Figures 4.A and 4.B. The product was analyzed using the TLC method and resulted in several types of oligosaccharides, such as mannose, manno-1,6-bisectriose, and mannotetraose, as the main product.

The oligosaccharide production from porang substrate showed the thin spot result because porang was a raw material that was more difficult to hydrolyze than a commercial substrate that was already pure. The experiment showed that the hydrolysis process by M8 continuously produced various oligosaccharide products in

different reaction times and continued to increase until it completely degraded in short times.

The hydrolysis of cellulose sources such as bagasse, porang, and konjac glucomannan by Cellulase C9 is shown in Figure 5. The product was analyzed using the TLC method and resulted in a thin chromatogram or almost spotless. It is because bagasse and porang were still raw materials. On konjac glucomannan, it resulted from thin spots of cellulose compound.

The hydrolysis of xylan sources such as xylan birchwood, EFB, and bagasse by xylanase X4 is shown in Figure 6. The products were identified as xylo-tetraose, xylo-pentaose, and xylo-hexaose, which were the main products of the hydrolysis process.

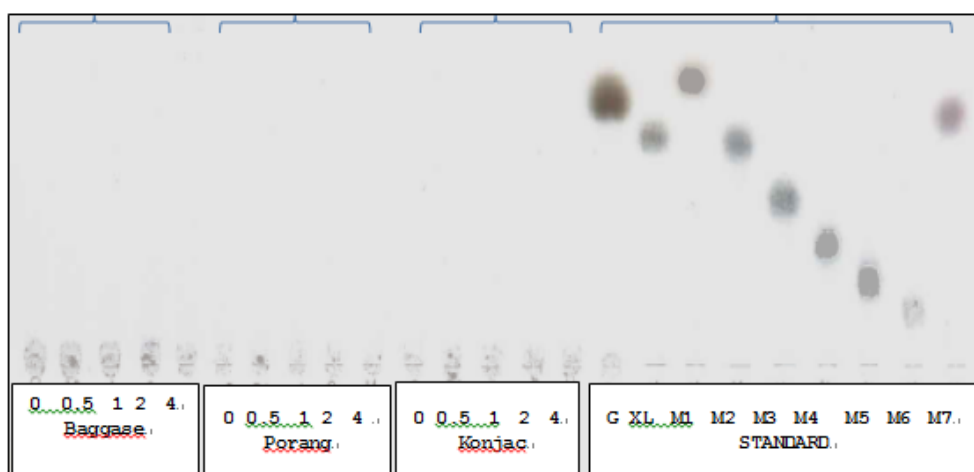


Figure 5. Thin layer chromatography analysis of hydrolysis products of cellulase C9 using Carboxymethylcellulose, Bagasse, EFB as a substrates. Standards (STD): glucose (G), xylose (M2), mannitriose (M3), mannotetraose (M4), mannopentaose (M5) and mannohexaose (M6). Substrate: enzyme (v/v) = 1: 10 and 1: 5, Substrate concentration 0.5% in 50 mM sodium phosphate buffer (pH 6.0), reaction time (h): 0, 0.5, 1, 2, 3 and 4 at 30°C condition

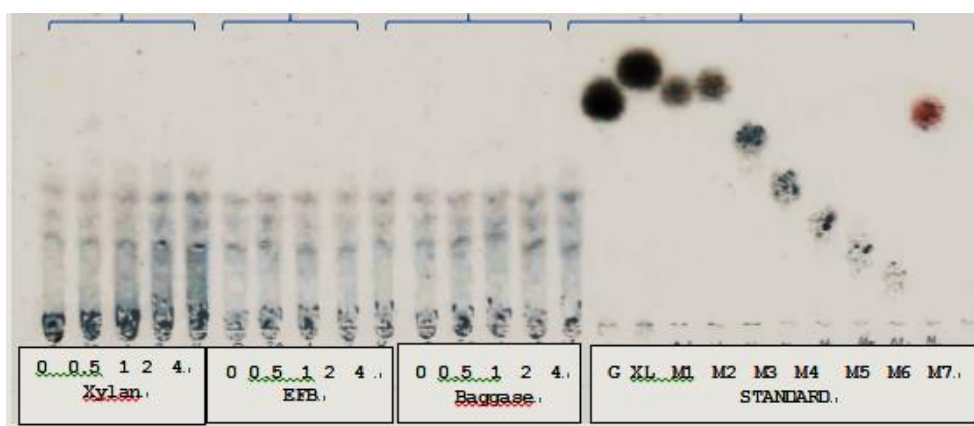


Figure 6. Thin layer chromatography analysis of hydrolysis products of xylanase X4 using xylan, EFB, Bagasse as a substrates. Standards (STD): glucose (G), xylose (M2), mannitriose (M3), mannotetraose (M4), mannopentaose (M5) and mannohexaose (M6). Substrate: enzyme (v/v) = 1: 10 and 1: 5, Substrate concentration 0.5% in 50 mM sodium phosphate buffer (pH 6.0), reaction time (hours): 0, 0.50, 1, 2, 3 and 4 at 30°C

Discussion

The screening method uses congo red staining application by adding various substrates, i.e., CMC for cellulose, locust bean galactomannan substrate medium for mannanase, and xylan for xylanase, into ASW medium abilities to excrete enzyme in substrates are shown. The clear zone area that appears at the medium surface shows the positive activity of converting substrate (polymer) into specific derivate sugar by an enzyme from potential isolate for each substrate. The oligosaccharide production from locust bean gum as mannan source results in mannose, mannobiose, mannotriose, and mannotetraose. It means that mannanase from *Bacillus subtilis* has two enzyme types, exoenzyme, and endoenzyme, for polymer hydrolysis. Hydrolysis of xylan sources such as xylan birchwood, EFB, and baggase by *Bacillus tequilensis* results in xyloetraose, xylopetaose, and xylohexaose as the main product of the hydrolysis process, and it means *Bacillus tequilensis* bacteria belongs to exoenzyme type. Cellulase production from *Bacillus cereus* shows weak activity in raw biomasses.

In conclusion, the isolation of xylanolytic, mannolytic, and cellulolytic marine bacteria has been conducted from 20 isolates. It has been able to identify three potential isolates based on 16S rDNA, and this process resulted in M8 (*Bacillus subtilis*), X4 (*Bacillus tequilensis*), and C9 (*Bacillus cereus*). Based on the TLC analysis, Mannanase M8 and Xylanase X4 had the potential to produce oligosaccharides with the size around tri-hexasaccharide as the main product.

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