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Dari: Journal of Pure and Applied Microbiology (editor@microbiologyjournal.org)
Kepada: sumardi_bio@yahoo.co.id
Tanggal: Rabu, 17 Juni 2020 16.26 WIB

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Thanks for approving your manuscript Ref No.: JPAM/6179/2020 for publication, it will be published online by June 25, 2020. Will confirm the payment, once received in our bank account.

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I send Orcid IDs authors. Sumardi number IDs : 0000-0002-2773-7083
and Achmad Arifiyanto number IDs: 0000-0002-0224-6309

Other authors didn't have orcid IDs.

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Editorial Journal

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Reviewer Comments:

Line number	Reviewer's Comments/Suggestions
	Corrections are noted in the article.Please find the attachment in this mail.

It is basic work but not written properly. I have noted the corrections in the article and highlighted. In my opinion, abstract needs to change and corrections are noted in methodology,result and discussion. Appropriate discussion is not there so **revision is required** before publication.

Regards

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Message

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Thank you

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- The authors confirm that the article including text, figures and tables, has neither been published before, not it is under consideration elsewhere and also all authors have made substantial contribution to this article and have given final approval for this version to be submitted.

Mannanase *Presence in* Halotolerant *Bacillus* sp Isolated from Mangrove Ecosystem at Hanura Beach Lampung

Abstract(reconstruct)

Mannose and mannooligosaccharide acted as prebiotic that consumed by probiotic bacteria. Mannanase enzyme activity has different levels depending on the source. The objective research was to obtain eminent strains of *Bacillus* sp. in mannan degradation which can potentially be a probiotic. The study employed a completely randomized design using four concentration levels repeated six times.Halotolerant Bacteria were isolated from mangrove ecosystem at Hanura beach, TelukPandan, Pesawaran District in Lampung Province. They are grown on the sea water complete agar media. Mannan degradation isolates were then characterized to determine their character with a variety of tests, including resistance to pH, salt, and metal ions, pathogenicity, and determination of mannanase production duration.Thirty strains are found to grow at 3-6% salt content, and 9 of them have mannanolytic activity. They grow optimally at pH 7-10. Seven isolates were proven to be positively hydrolyzed blood agar in the pathogenicity test. The addition of Iron (III) Chloride increased the enzyme activity by 11.12% in IBK3 isolates at 96 hours of cultivation period which was 0.05 UmL-1. It acted as cofactors of enzymatic reactions.Strains *Bacillus* sp., were able to degrade mannan substrate. It quantified using Index of mannanolytic. Strain IBK3 has the highest index of mannanolytic activity as much as 10.74. Their ability to grow in salt media indicated that they were halotolerant. They were more likely to live at base rather than acid habitat. Only IBK3 and ID2K1 showed non-pathogenic isolates. Only $FeCl_3$ addition has proven to rise up enzymatic activity.

Keywords: Characterization, *Bacillus sp.,* Mannanase, Probiotic

INTRODUCTION

Mangrove is a coastal intertidal wetland forest composed of halophytic tree and shrub species [1] and Indonesia has the largest mangrove forest in Southeast Asia [2]. The area of mangrove forests in Lampung Province was 17.110 ha [3]. Hanura Village, TelukPandan District, Lampung was one of the many Lampung regions that had a mangrove ecosystem. The coastal waters of Hanura Village were mostly overgrown with mangrove forests and make them fertile because they get an accumulation of organic material from marine aquaculture activities in the form of sediment residues and mangrove leaf litter on the forest floor [4]. Organic materials from litter and dead mangrove stems are materials that decomposed by microorganisms. The microorganisms produce minerals that help maintain the fertility of the surrounding soil [5]. Structural analysis of the polysaccharides in the cell walls of dicotyledon is known to consist of cellulose, hemicellulose, and lignin. [6].

Hemicellulose was the second polysaccharide which was very abundant in nature after cellulose. Hemicellulose had two main components, namely hetero-1.4-β-D-xilan and hetero-1.4-β-D-mannan [7].The mannan component breaks down by mannanase into mannose and mannooligosaccharide. Mannose and mannooligosaccharide acted as prebiotic that consumed by probiotic bacteria. Mannanase enzyme activity has different levels depending on the source. This enzyme can be found from various sources including animals, plants, and microorganisms. In research, generally mannanase is taken from microorganism because it can be produced in large quantities and the isolation process is also easier [8]. Mannanase producing microorganisms was include *Bacillus subtilis* MAN-511 [9], *Bacillus subtilis* TJ-102 [10], *Bacillus pumilus* M27 [11] and *Bacillus cereus* N1 [12]. It would be very good if these bacteria are also probiotic. Probiotic bacteria produce enzymes that are able to break down complex compounds to be simple so they

are ready to be used by fish feed [13]. The type of probiotic bacteria is influenced by the environment. Environmental factors such as pH condition and salinity where the bacteria are isolated greatly affect their ability to grow, develop and to carry out their functions as expected [14]. The ability of microorganisms to degrade mannan was very helpful in the field of aquaculture. It applied to the media for shrimp maintenance, water media, sub grade and feed. This study aims to obtain eminent strains of *Bacillus* sp. in mannan degradation which can potentially be a probiotic candidate for shrimp farming.

MATERIAL AND METHODS (INCULUDE THE REFERENCE)

Composition media

Bacteria were cultured at Sea Water Complete (SWC) media with a composition of 0.5% locust bean gum, 0.5% peptone, 0.1% yeast extract, 0.3% glycerol, 1.5% agar, 25% aquades, and 75% sea water.

Bacteria Selection for Mannanolytic activity(Reference)

Bacteria selection is conducted by qualitative method using agar plate assay. Bacterial collection were grown on SWC agar with the addition of 0.5% locust bean gum (LBG). After incubation for 24 hours, the colonies were stained with 1% congo red for 15 minutes.It washed again using 1M NaCl. Observations were made included mannanolytic index and bacterial staining. Selected colony with highest clear zones was tested using enzymatic assay with DNS method to determine the length of incubation time for enzyme production.

Salt tolerance test

Mannanolytic isolates were further tested for their resistance to salt content. Bacteria were grown on SWC agar with the addition of salt as much as 0%, 3%, and 6%. The size of the colony was observed after an incubation period of 24 hours at room temperature.(note NaCl)

pH tolerance test

Mannanolytic isolates were tested for their resistance to media pH stress. Bacteria were grown on SWC agar media with pH 4, 7, and 10. After an incubation period of 24 hours at room temperature, growth and size of the colonies were observed and recorded. **(note what chemical are used ,in which normality?)**

Pathogenicity test

Mannanolytic bacterial isolates were tested for their pathogenicity using blood agar SWC media. Observations were made after an incubation period of 24 hours at room temperature. Isolates that have the ability to hemolysis are shown by the formation of clear zones around the colony**.(note human blood or sheep blood)**

Metal susceptibility test

Selected isolates that did not show any pathogenicity in the previous test were further tested in determining the mannanolytic index by growing on SWC media with the addition of metal substrates and ions to know mannanase acivity in metal ion present. The metals used are Fe, Pb, Cu and Al in the form of salt $FeCl_3$, $PbCl_2$, $CuCl_2$, and $AlCl_3$.

Mannanase enzyme production

Starter was constructed using 50 mL liquid SWC media on 250 mL Erlenmeyer. The starter was incubated at room temperature for 48 hours using an orbital shaker. Furthermore, the starter was inoculated as much as 5 mL into 45 mL of liquid SWC media with the addition of 0.5% locust bean gum substrate and incubated in the shaker orbital.**(Note the ___rpm)**

Enzyme production

Determination of the length of production time proposed by taking 5 mL of culture every 24 hours of incubation for 7 days. It centrifuged at 8500 rpm for 15 minutes. Enzyme activity was determined using 3.5-dinitrosalicylic acid (DNS) method and the absorbance was read at a wavelength of 540 nm. One unit (IU) of -mannanase activity was defined as the amount of protein producing 1 μmol/L of reducing sugar per minute (e.g., mannose) under standard conditions.

RESULTS

Bacteria Selection for Mannanolytic activity

The selection results showed that there were 9 mananolytic isolates from a total of 30 isolates tested. These isolates were IAK1, IAK2, IAK4, IBK3, IDK6, ID2K1, ID2K2, IKK1, and IKK3. The IBK3 isolate gave the largest mananolytic index of 10.74 which was indicated by the clear zone formed around the colony (Table 1). **Gram staining indicated that IBK3 isolates were gram-negative bacteria in bacilli form.(Add gram's reaction profile)(Is the Bacillus sp. [noted in conclusion] gram negative rods?)**

Table 1. Bacteria Selection and Characterization

No.	Isolate	Colony				Mannanolytic index
		Shape	Margin	Elevation	Color	
1	IAK1	Circular	Filamentous	Raised	White	5,35
2	IAK2	Circular	Entire	Raised	White	6,57
3	IAK3	Irregular	Lobate	Raised	Yellow	-
4	IAK4	Circular	Filamentous	Raised	White	5,47
5	IA2K1	Circular	Entire	Raised	White	-
6	IA2K2	Circular	Entire	Flat	Pink	-
7	IA2K3	Circular	Filamentous	Flat	Clear	-
8	IA2K4	Circular	Entire	flat	Clear	-
9	ILK3	Circular	Entire	Raised	Orange	-
10	ILK5	Circular	Entire	Raised	Orange	-
11	ILK6	Circular	Undulate	Raised	Pink	-
12	ILK9	Irregular	Lobate	Flat	Clear	-
13	IL2K5	Circular	Entire	Flat	Clear	-
14	IL2K8	Irregular	Undulate	Flat	Clear	-
15	IL2K9	Circular	Entire	raised	White	-
16	IKK1	Circular	Undulate	Raised	White	4,96
17	IKK3	Circular	Entire	Raised	White	2,61
18	IDK4	Circular	Entire	Flat	White	-
19	IDK6	Circular	Entire	Raised	White	9,14
20	ID2K1	Circular	Filamentous	Flat	White	10,64
21	ID2K2	Circular	Undulate	Raised	White	4,18
22	ID2K3	Circular	Entire	Raised	Cream	-
23	IBK1	Circular	Filamentous	Flat	Clear	-
24	IBK3	Circular	Entire	Raised	Cream	10,74
25	IPK3	Circular	Irregular	Flat	Clear	-
26	KA2K2	Irregular	Undulate	Raised	Pink	-
27	KA2K4	Circular	Entire	Convex	Pink	-
28	KBK2	Irregular	Undulate	Raised	White	-
29	KLK7	Irregular	Undulate	Raised	White	-
30	KL2K2	Irregular	Lobate	Flat	White	-
Number	30	30	30	30	30	9

Salt tolerance test

Probiotic microbes were able to grow on bile salts and living in colonies [16]. The results showed that salt levels can affect bacterial growth. It is indicated by the size of the colony which varies in 0%, 3%, and 6% salt content. Colony size of some bacteria tends to be larger in media with salt stress 0% and 3%, whereas in media with salt content of 6% the size of the colony tended to be smaller (Figure 1).

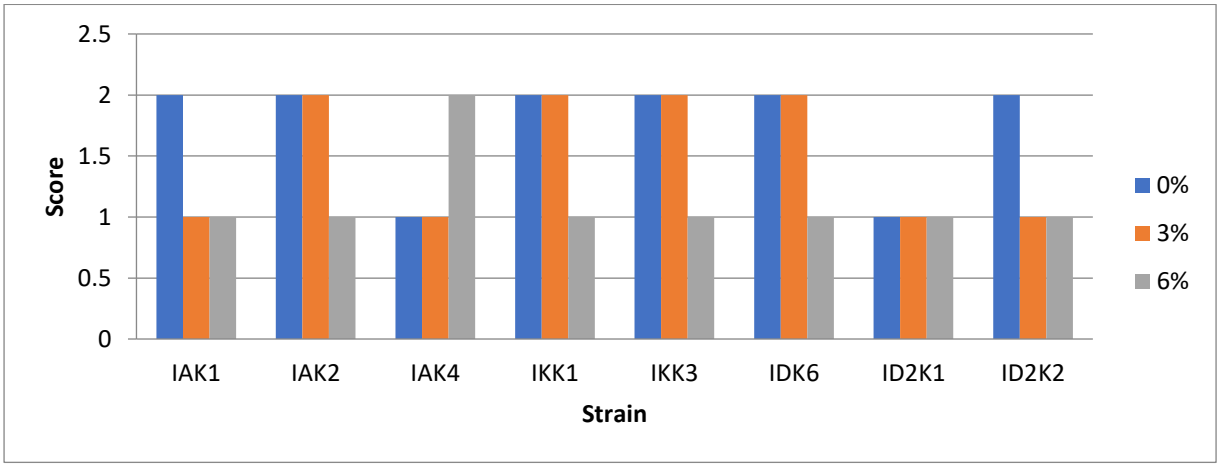

Figure 1. Bacterial salt tolerant assay

Description: Score null = none growth, Score1 = small colony, Score 2 = Large colony,

pH tolerance test

In testing the pH of the media showed that all isolates resistant to alkaline stress but not resistant to acids, this was indicated by the absence of appearance of bacteria that grow on media with a pH of 4 (Figure2).

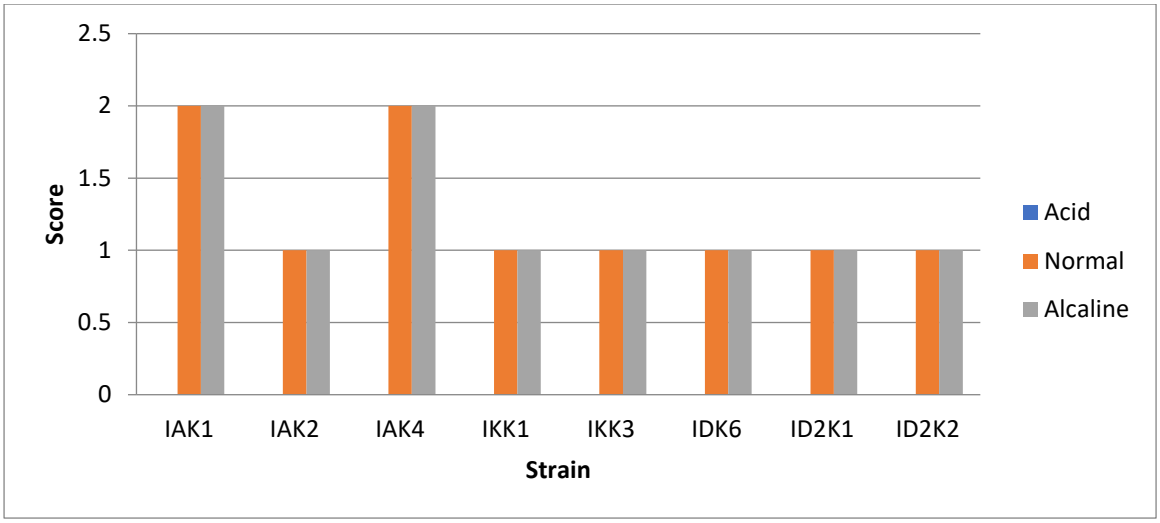

Figure 2. Bacterial pH tolerant assay

Description: Score null = none growth, Score1 = small colony, Score 2 = Large colony

Pathogenicity test

Isolates that show beta hemolysis or total hemolysis were IAK2, IDK6, ID2K2, IKK1, and IKK3. Isolates included in gamma hemolysis were IBK3 and ID2K1 (Figure 3). Isolates which did not show hemolysis were used for further testing because they were not pathogenic bacteria.**(rewrite the content)**

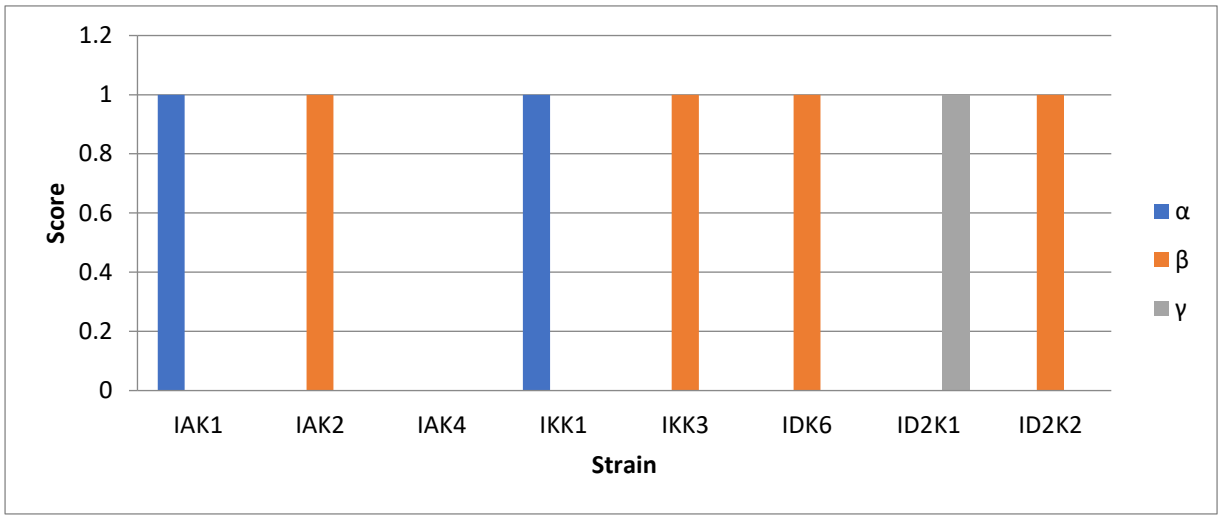

Figure 3. Hemolytic test using blood agar assay

Description: Score null = negative reaction, Score1 = positive reaction

Metal susceptibility test

The presence of metal ions in the media acted as inhibitors or as cofactors of enzymatic reactions. The addition of $FeCl_3$ metal as much as 20mM increased the mannanolytic index of IBK3 isolates which was 3.76 or 11.12% higher when compared to controls (Figure4).

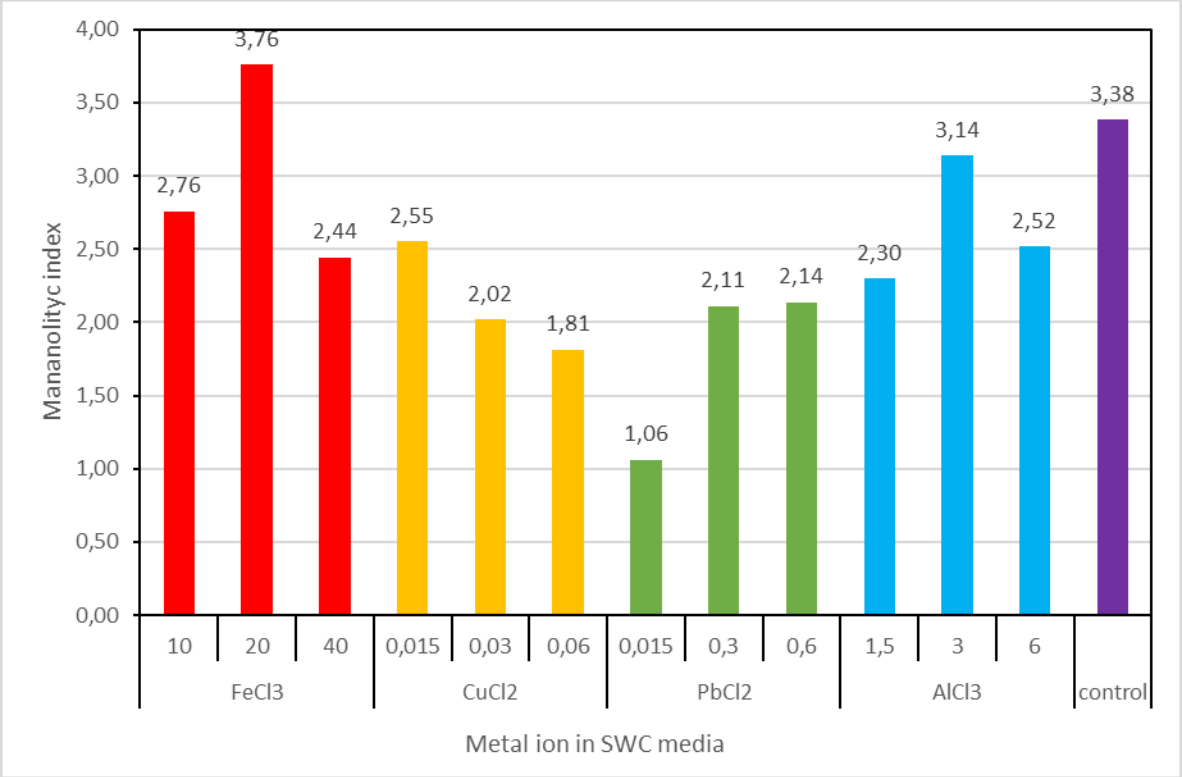

Figure 4. Metal effect of $FeCl_3$ (10mM, 20mM, 40mM), $CuCl_2$ (0.015mM, 0.03mM, 0.06mM), $PbCl_2$ (0.015mM, 0.03mM, 0.06mM), and $AlCl_3$ (1.5mM, 3mM, 6mM) to IBK_3isolate in producing mannanase enzyme.

Enzyme production

Mannanase enzymes are primary metabolites needed by bacteria to degrade carbon sources in the form of mannan contained in the locust bean gum substrate during bacterial growth. One unit of mannanase activity was defined as the amount of enzyme that released 1 $\mu.mol^{-1}$ of reducing sugar per min under standard assay conditions [22]. The IBK3 isolate showed the highest enzyme activity at the production time of 96 hours, which was 0.05 UmL-1 and continued to decline until 168 hours (Figure 5).

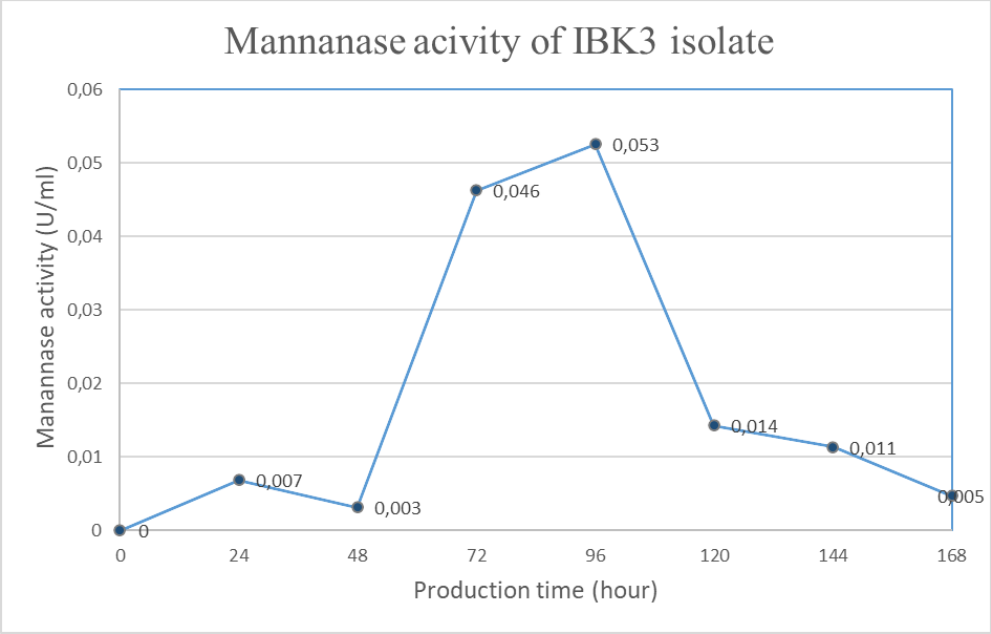

Figure5. IBK3 isolate mananase activity curve determined using DNS method and analyzed using spectrophotometer at λ 540nm.

DISCUSSIONS

Isolation of bacteria on SWC agar media with the addition of 0.5% locust bean gum (LBG) substrate showed the growth of mananolytic bacteria. The presence of mananolytic bacteria indicated by the presence of clear zones formed around bacterial colonies after staining with congo red and NaCl. Congo red was bound into the ß-1.4-D-manopiranosil bond and the media to turn red. LBG contained in the media had broken down by bacteria as a source of carbon in the process of metabolism. The breakdown produced mannose monosaccharides. Mannose hadn't a β-1.4-D-manopiranosil bond. It caused congo red unable to colorize the area around the colony and causes the formation of clear zones [15].(rewrite the content)

The ability of isolates to grew in stress levels of salt influenced by the source of isolates obtained that our isolate was taken from estuary ecosystem. These results were almost the same as the

research of [17], they were show that marine bacteria can grow well at 2.5 - 5% salt content.Bacteria need nutrients to grow during the incubation period [16]. Nutrients were obtained from the surrounding environment, in this case the media. The more acidic in media caused more hydrogen content in more media. The presence of too many hydrogen ions in the media inhibited transport nutrients.

Probiotic agents must not be pathogenic and had proven to have health effects [18]. The observation of hemolysis test gave of various results. Hemolysis divided into 3 was namely alpha, beta, and gamma hemolysis. Beta hemolysis was a complete lysis of red blood cells, it caused around the colony turn to clear area. Alpha hemolysis was partial hemolysis while gamma hemolysis was no hemolysis [19].(**Discuss with obtained result)**

Mannanase activity of *Bacillus subtilis* increased with the addition of Fe^{3+} metal ions[20]. Unfortunately it is unable tolerated Cu_2^{+} more than 0.015mM, because [7] confirmed that Cu^{2+} acted as an inhibitor of the mannanase enzyme from *Geobacillus stearothermophillus* L-07. It tolerated lead (Pb^{2+}) less than 0.06 mM. It accumulated Al^{3+} in maximum no more than 3 mM. Beside that metal ions played as inhibitors when it bound to the active side of the enzyme so that the enzyme became ineffective in binding the substrate (Figure 1).These results were in accordance with the research [21] which showed the highest activity of the mannanase used *Bacillus cereus* in the production time of 88-96 hours. It [7] showed the highest activity of the enzyme mannanase isolate *Geobacillus stearothermophillus* L-07 at the time of production of 36 hours with 3.1 $U.mg^{-1}$activity.

CONCLUSION

Strains *Bacillus* sp., were able to degrade mannan substrate. It quantified using Index of mannanolytic. Strain IBK3 has the highest index of mannanolytic activity as much as 10.74. Their ability to grow in salt media indicated that they were halotolerant, it because they were isolated from mangrove ecosystem at Hanura Beach Lampung Province. They were more likely live at base rather than acid habitat. Only IBK3 and ID2K1 showed non-pathogenic isolates. Only $FeCl_3$ addition has proven to rise up enzymatic activity.

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