ISSN: 2277-3754
ISO 9001:2008 Certified
International Journal of Engineering and Innovative Technology (IJEIT)
Volume 8, Issue 10, April 2019

A suitable condition for producing mannanase enzyme activity By *Bacillus subtilis*P2-5 and agricultural wastes using general factorial experiment

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Abstract: ***The agricultural product is one of the core business sectors in Thailand since it makes huge incomes for the country. However, transformation processes of agricultural products generate various wastes and these wastes generate disposal problems to concerned areas. This research aims to use some wastes such as coffee ground, tea waste, and copra meal for producing a useful enzyme called mannanase which is used in many biochemical processes mostly in food and detergent industries. There are four concerned factors, which are waste sources, inoculum percentages, temperature and speed. Since each of which has multi-level, a general factorial experiment is then conducted to study the effect of each factor and also to determine a suitable condition of the factors to obtain the maximum enzyme activity. The result shows that using 1%(v/v) of inoculum, 37 ℃, and 200 rpm, tea waste obtains higher mannanase enzyme activity than coffee ground and copra meal. Although, the level of mannanase enzyme activity obtained from tea waste is significant lower than using a commercial source called locust bean gum (LBG), but we can use the tea waste for producing the mannanase enzyme activity for free instead of buying LBG. The contribution of this research is not only to reduce the mannanase production cost but also be a guideline for waste management strategy.***

Keywords: **Agricultural waste, Mannanase enzyme activity, Design of Experiment.**

I. INTRODUCTION

Agricultural sector is one of the main economic growths in Thailand because the agricultural area in Thailand is approximately 186.6 million square kilometers, which is 36.3 % of the total area. This area provides raw materials to produce agricultural products, such as vegetable and fruit frozen, food, and beverage. However, we also have various wastes from this sector, for example, palm waste, wheat bran; saw dust corn cobs, coffee waste, copra meal, and rice husk. These wastes generate the disposal problems, which is one of the biggest problems in Thailand. Therefore, the existing approach to reduce this problem is the waste management system, which is to recycling or reusing in order to improve environment condition. The waste management system concept (USDA,2011) includes six basic functions, which are production, collection, storage, treatment, transfer and utilization as shown in Fig.1.

Transformation process is generating waste so disposal strategy need to applied base on sustainable development factor. Waste from agricultural and food process can convert by physical, chemical and biological method. The biological method focus on waste disposed by utilization, treatment and recycling, which produce new product or value added for instance, an enzyme from fermentation process (Raveendran et.al,2016).The market of industrial enzymes forecasting increase to $6.30 billion in 2021 at a compound annual growth rate and can be classified into four application sections: food industry, animal feed industry, detergent industry, and technical industry as shown in Fig. 2 (BCC research report,2017) .Nowadays, Thailand has waste materials such as coffee grounds, tea residue and copra

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meal etc. These wastes containing mainly mannan components, it is interesting to use as a raw material for mannanase enzyme production, which increases the value of waste materials.

Mannan is the major constituent of many plants such as ivory nut, locust bean, guar gum, konjac, palm kernel, coffee seed, and copra meal. Mannanase are enzyme that can randomly hydrolyze the 1,4-beta-D-mannosidic linkages in the main chain of mannans and heteromannans (McCleary,2012). Mannanase is the most important enzyme, which is produced by microorganisms, including bacteria and fungi such as *Bacillus* sp., *Aspergillis* sp. Phothichitto*et al*., isolated *Bacillus circulans* NT 6.7 from soil, which show high mannanase activity of 0.306 U/mL. The optimal conditions for mannanase production from *Bacillus circulans* NT 6.7 are at pH 6.0, temperature 45°C with locust bean gum (LBG) as a carbon source (Phothichitto,2006). Chantorn et al. report that the mannanase enzyme is produced by *Bacillus* sp. GA2 (1) and *Bacillus* sp. GA1(6) using corn cob as carbon source. The result found that mannanase enzyme had 0.53 and 0.25 Unit/mL, respectively (Chantorn et al,2016).Chantorn et al. produce mannanase by *Penicilliumoxalicum* KUB-SN2-1 using coffee residues and shaking incubator at 30°C with rotation speed of 200 rpm. The specific mannanase activity highest at 16.21 (U/mg protein) (Chantorn et al, 2016).

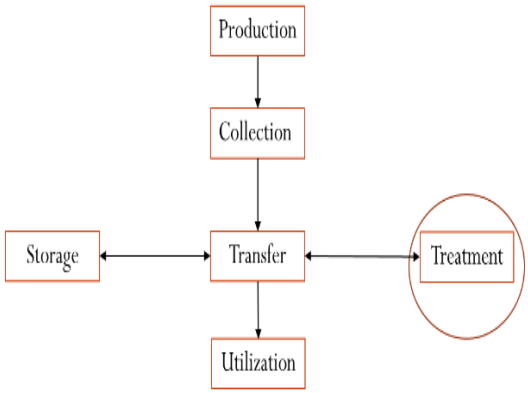

Fig.1 Waste management system concept

Source: Agricultural Waste Management Field Handbook, 2011

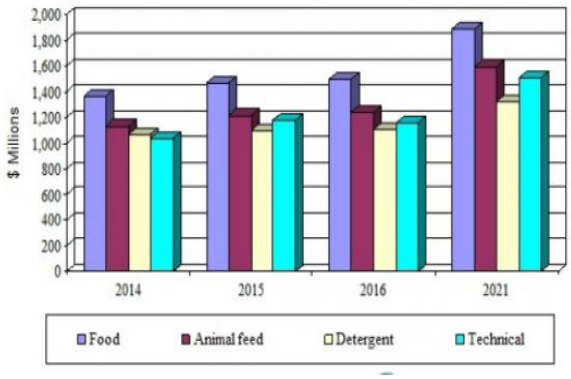

Fig.2 Global market for Industrial enzymes separate by type 2014-2021 ($Million)

Source: A BCC Research Biotechnology, 2017

This research aims to recycling wastes source called coffee ground, tea waste, and copra meal by using them as a carbon source and use *Bacillus subtilis* P2-5 as an inoculum for producing the mannanase enzyme. It is not only to study the effects from the mentioned wastes and inoculum but also to determine a suitable condition to produce the maximum mannanase enzyme activity.

II. MATERIAL AND METHOD

A. Microorganism and Inoculum preparation

*Bacillus subtilis*P2-5 is isolated from soil in Pathumthani Provience, Thailand (Pangsri, 2016) and stored at -20ºC on nutrient broth at the Biotechnology Program, Valaya Alongkorn Rajabhat University under the Royal Patronage Pathumthani Province, Thailand. *B. subtilis* P2-5 is transferred into nutrient broth medium and shaken at 150 rpm for 18 hours at 37ºC, which is used for inoculum.

B. Materials Preparation

Locust bean gum (LBG) is purchased from Sigma Chemical, USA. All other chemicals used are of analytical grade. Preparation of three wastes including coffee ground, tea waste and copra meal are used as carbon source for mannanase enzyme production. They are dried at 60°C for 48 hours. After that, the coffee ground, tea waste and copra meal are blended and milled to 0.5 mm in size as shown in Fig.3.

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Fig. 3 Waste sources consisted of LBG, Coffee ground, Tea waste and Copra meal

C. Enzyme Production

The enzyme is produced in an 250 ml Erlenmeyer flask containing 100 ml of medium (%w/v): 0.4 bacto-peptone, 0.3 KH_2PO_4, 0.2 K_2HPO_4, 0.05 $MgSO_4$.7H O, 0.0002 $CoCl_2.6H_2O$, 0.0005 $FeSO_4.7H_2O$, 0.0002 $MnSO_4.H_2O$, and 0.0016 $ZnCl_2$. The *Bacillus subtilis* P2-5 grown with OD 600 of 0.5 is inoculated. The 1 and 5% (v/v) cultured of *Bacillus subtilis* P2-5 are transferred into the producing medium (PM), which contains 1% (w/v) supplementation of additional substrate (LBG, coffee ground, tea waste, and copra meal) and shakes at 150 and 200 rpm for 24 hours at 37 and 45 ºC. The cell suspension is centrifuged at 7,000 rpmfor 20 min at 4ºC, and the crude mannanase are collected and stored at -20ºC for enzyme assay.

D. Determination of Mannanase Activity

Mannanase activity is measured at 50°C for 30 minutes using a reaction mixture containing 0.5 ml of crude enzyme samples and 0.5 ml of 0.1M phosphate buffer pH 7.0 with 1% (w/v) LBG. The amount of reducing sugar released is determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme producing 1 micromole of mannose per minute under experimental conditions.

III. EXPERIMENTAL DESIGN

There are four independent variables (factors) directly concerned to the mannanase enzyme activity, which are inoculum percentage, wastes, temperature, and speed. In this experiment, we select *Bacillus subtilis*P2-5 as the inoculum since it is one of the microorganisms obtaining high enzyme activity. For the wastes, there are three types of wastes, which are coffee ground, tea waste, and copra meal. The effects of these wastes are compared to the commercial carbon source called LBG. For the temperature and speed, we follow the equipment specifications in our laboratory. All levels of the factors are shown in Table 1. For the dependent variable or response, we measure the mannanase enzyme activity (U/ml) and use it as the response.

Table 1: Factors and levels

Factors	Level	Detail
Waste sources	4	LBG, coffee ground, tea waste, and copra meal
Inoculum percentage	2	1 and 5 % (v/v)
Temperature	2	37 and 45 ºC
Speed	2	**IV.** d 200 rpm

IV. RESULTS AND DISCUSSION

A. Effect of factors on mannanase enzyme activity

Based on the reduced model shown in Table 2, all main factors except speed, and some two-way interactions significantly affect to the mannanase enzyme activity. From the interaction graphs shown in Fig. 4, they show that LBG (commercial carbon source) obtains the highest enzyme activity compared to other types of wastes. This happens for all combinations among inoculum percentage, temperature, and speed. It also observes that the tea waste obtains higher enzyme activity than coffee ground and copra meal. The mannanase enzyme activity is produced very well in higher speed and lower inoculum percentage and temperature environment.

Table 2: Analysis of Variance (ANOVA)

Source	Degree of freedom	Adj SS	Adj MS	F value	p-value
Model	13	8.33377	0.64106	80.20	0.000
Linear	6	7.8125	1.3020	162.9	0.000

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ISO 9001:2008 Certified
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		6	9	0	
Waste source	3	7.5582	2.5194	315.2	0.000
		6	2	0	
%Inoculum	1	0.1084	2.5194	13.57	0.002
		8	2		
Temperature	1	0.1457	0.1457	18.24	0.000
		6	6		
Speed	1	0.0000	0.1457	0.01	0.932
		6	6		
2-Way	7	0.5212	0.1457	9.32	0.000
Interactions		2	6		
Waste	3	0.2256	0.0752	9.41	0.001
source*%Inocul		8	3		
um					
Waste	3	0.2564	0.0854	10.69	0.000
source*Temp			8		
Temp*Speed	1	0.0390	0.0390	4.89	0.040
		9	9		
Error	18	0.1438	0.0079		
		7	9		
Total	31	8.4776			
		5			

		R-Sq(adj) =
S = 0.0894038	R-Sq = 98.30%	97.08%

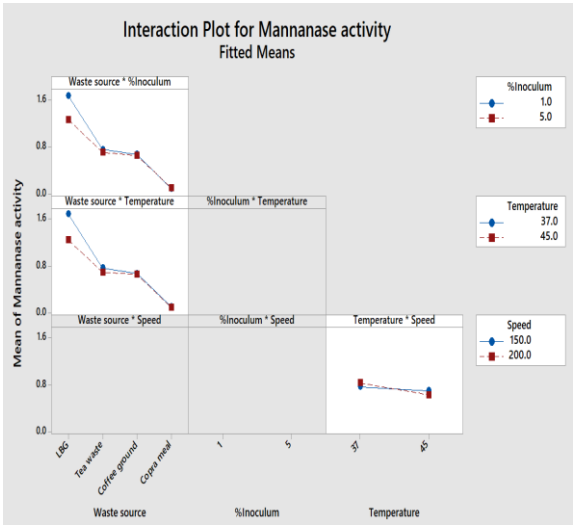

Fig.4. Interaction plots

B. The regression equation and validation of the optimum condition

Based on a regression analysis, the relationship between factors and mannanase enzyme activity can be shown in Eq. (1) to (4). Note that these equations can predict the variation up to 97.08% as shown at the end of Table II (R-sq (adj)).

LBG: Mannanese activity = 1.53
- 0.1013 Inoculum percentage + 0.0059 Temperature
+ 0.01427 Speed - 0.000350 Temperature*Speed (1)

Tea waste: Mannanese activity = -1.35
- 0.0126 Inoculum percentage
+ 0.0517 Temperature+ 0.01427 Speed
- 0.000350 Temperature*Speed (2)

Coffee ground: Mannanese activity = -1.75
- 0.0048 Inoculum percentage
+ 0.0594 Temperature+ 0.01427 Speed
- 0.000350 Temperature*Speed (3)

Copra meal: Mannanese activity = -2.36
+ 0.0022 Inoculum percentage
+ 0.0601 Temperature+ 0.01427 Speed
- 0.000350 Temperature*Speed (4)

C. A suitable condition and confirmation run

Based on Eq. (1) to (4), we can use an optimization tool in Minitab 17 to determine a suitable condition for producing the maximum mannanase enzyme activity, and the suitable condition is to use tea waste with 1% of inoculum, 37 ºC, and 200 rpm. This condition obtains the highest mannanase enzyme activity of 0.8243 U/mL as shown in Fig. 5. Note that LBG is not considered since it is the benchmark for this study.

To ensure that the suitable condition works properly, 30 samples are collected based on this condition and its mean is tested based on the hypotheses below:

H_0: μ = 0.8243

H_1: μ> 0.8243

From one sample *t*-test shown in Fig. 6, the result shows

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that the suitable condition obtained from our experiment works very well (reject H_0 since p-value < 0.05). It produces the mannanase enzyme activity of 0.83845 U/mL, which is better than our prediction.

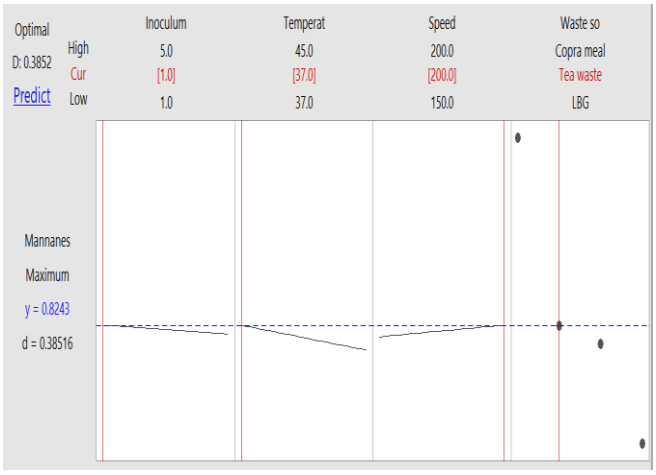

Fig. 5. A suitable condition for producing mannanase enzyme activity

One-Sample T: Confirm run

Test of μ = 0.8243 vs > 0.8243

Variable	N	Mean	StDev	SE Mean	95% Lower Bound	T	P
Confirm run	30	0.83845	0.03181	0.00581	0.82858	2.44	0.011

Fig. 6. Result of one sample *t*-test

V. CONCLUSION

In this research, three types of wastes and Bacillus subtilisP2-5are used to produce the mannanase enzyme activity in various temperature and speed conditions. The result shows that tea waste obtains higher enzyme activity than coffee ground and copra meal. Although, the level of mannanase enzyme activity obtained from tea waste is significant lower than using a commercial carbon source (LBG) around 50%, but we can use the tea waste for producing the mannanase enzyme activity for free instead of buying LBG. The contribution of this research is not only to reduce the mannanase production cost but also be a guideline for waste management strategy.

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