

# Amylase Producing *Bacillus megaterium* T04 Isolated in Rach Lang Stream of Vietnam

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## ABSTRACT

In the study, *Bacillus megaterium* T04 isolated from Rach Lang stream in Vietnam was identified. The stream samples were diluted in 0.9 % NaCl broth and then spread onto the ISP4 supplemented with rice and wheat starch. The colonies showed the strongest hydrolyzing activity were picked up and identified by biochemical test and 16S rRNA sequencing analysis. The starch hydrolyzing ability of this strain was detected by starch agar plate method. For maximum  $\alpha$ -Amylase production, including 174.7 IU/ml and 0.2 IU/ml in medium containing wheat and rice, respectively was obtained after 72 h of incubation. The enzyme still showed high activity in 60% ammonium sulfate that was necessary for study on the enzyme characteristics. As a result, *Bacillus megaterium* T04 could produce high yield of amylase in simple conditions.

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## INTRODUCTION

Microorganisms contribute to degradation of acid orange dye (Maulin *et al.*, 2014), persistent insecticides (Selvanayagam and Vijaya, (1989), and are capable of metabolizing the herbicide metolachlor (Saxena *et al.*, 2010). Microorganisms can produce many kinds of products such as lipase (Sekhon *et al.*, 2003), poly (3-hydroxybutyrate) (Rodriguez-Contreras, 2013), potential proteins (Vary *et al.*, 2007). Among their products, amylases are hydrolases than can breakdown or hydrolysis starch. Amylases can be applied in pharmaceutical fields (Kandra, 2003; Pandey, 2000). Amylases were produced in yeasts, molds, bacteria (Roopa and Sushma, 2013; Vihinen and Mantsala, 1989; Buzuhi and Martini, 2002). However, many previous studies showed that amylases produced in bacteria were more thermostable than fungal amylases (Omemu *et al.*, 2009). Moreover, *Bacillus* strains were focused to produce amylases because they can grow quickly and require the simple nutrients for amylase production that could be benefit for a developing country. There was *Bacillus subtilis* IP 5832

(Nataša *et al.*, 2011) producing amylases in in shake flask and laboratory fermenter batch cultures. *Bacillus sp.* PN5 could produce a highly thermostable and alkaline amylase (Saxena *et al.*, 2007). A moderately thermophilic *Bacillus subtilis* strain for starch processing was also studied (Asgher *et al.*, 2007). *Bacillus sp.* IMD 435 produced the raw starch-digesting  $\alpha$ -amylase (Hamilton *et al.*, 1999). *Bacillus sp.* I-3 could produce thermostable  $\alpha$ -amylase and its use in the direct hydrolysis of raw potato starch (Goyal *et al.*, 2005). *Bacillus caldolyticus* produced  $\alpha$ -amylase in complex media (Schwab *et al.*, 2009) while *Bacillus licheniformis* using an economical medium to produce  $\alpha$ -amylase. Amylase production in bacteria could be obtained under different conditions such as solid fermentation for *B. amyloliquefaciens* (Tanyildizi *et al.*, 2007) and *Bacillus cereus* (Vijayabaskar *et al.*, 2012). In this study, *B. megaterium* T04 was identified and determined the starch hydrolyzing ability due to find out a source for amylase production with high yield in simple nutrient.

## MATERIALS AND METHOD

### Isolation and identification of starch hydrolyzing bacteria

Rach Lang stream is a small stream belonging to Binh Thanh district, Hochiminh city, Vietnam), but it is polluted by many wastes containing cellulose, starches. Therefore, Rach Lang stream was thought as a source of amylase producing micro-

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organism. In this study, approximately 1 L of Rach Lang stream was collected and transferred to the lab. The stream samples were diluted in 0.9% NaCl broth and then spread onto the ISP4 media supplemented with rice starch or wheat starch for starch hydrolyzing detection. No specific permit was required for the described field studies. Samples were incubated for 48 hours at room temperature. Then, colony showing the strong hydrolyzation were selected for identification. The pure colony was identified by biochemical characterization based on the ability of the isolation to utilize different carbon sources, which determined by API 50CHB (bioMerieux, Lyon, France) and 16S rRNA sequencing analysis. Because of the purpose of the study was to isolate *Bacillus megaterium*, the primers should be specific to *Bacillus megaterium*. Therefore, the forward primer was Ba1 (5'-AGTCGAGCGAACTGATTAGAAGCT -3') and the reverse primer was Ba 2 (5'-TACTCTCGTACTTGTCTTCCCTAACAACA-3'). The PCR reaction were performed as follows: 95 °C for 5 min; 30 cycles of 95 °C for 20 s, 50 °C for 20 s and 72 °C for 3 min; and a final extension at 72 °C for 10 min. The PCR product was stored at 4 °C. The PCR product was purified and sequenced. The homology comparison of the 16S rRNA sequences was performed using the NCBI BLAST database.

### Starch hydrolyzing ability test

#### Sample preparation

Cell free supernatant (CFS) of bacterial cultures in ISP4R and ISP4W medium at three temperatures (25, 37, 45 °C) in three incubation periods (24, 48, 72 hours) were assayed for their starch hydrolyzing ability. The CFS from cultures incubated at 37°C for 48 hours was chosen to assay the enzyme precipitation capability by ammonium sulfate precipitation method (Ammar, 2004). 50 mL of the CFS were added with ammonium sulfate to 40% saturation. The mixtures were centrifuged at 5000 rpm for 15 min at 4 °C to collect the precipitated proteins. The pellets were dissolved in distilled water. The left supernatant was concentrated again with ammonium sulfate to 60% saturation. Each resulted fraction was assayed separately for its ability to hydrolyze starch.

#### Starch hydrolyzing test

Rice starch and wheat starch were used as substrates for the activity tests. Enzyme  $\alpha$ -amylase (Sigma) was used to make standard curve to represent the relation between the diameter of the halo zones and enzyme concentration. Ammonium sulfate was used to concentrate the enzyme. Lugol's iodine solution was used to visualize the clear zone through the formation of iodine-starch complex. Starch agar plate method was done to assay the starch hydrolyzing ability of *Bacillus megaterium* T04 (Farias *et al.*, 2010). Rice starch containing agar plates (1% rice starch) and wheat starch containing agar plates (1% wheat starch) were used to detect the activities in ISP<sub>4R</sub> and ISP<sub>4W</sub> medium respectively. Both CFS of *Bacillus megaterium* T04 cultures and ammonium sulfate fractions (in 40% and 60% saturation) were tested. The 11-millimeter-in-diameter wells were made on the starch agar plates

using sterile 11 mm cork borer. A volume of 300  $\mu$ L CFS was pipetted into each well. All the plates were placed overnight at room temperature. On the next day, they were stained with Lugol's iodine solution to visualize the halo diffusion zones. The results were recorded by observation and measurement the diameter of the halo zones (in mm) around the wells. The same assay was performed to enzyme  $\alpha$ -amylase (Sigma) at different concentration (10 mg/mL, 5 mg/mL, 1 mg/mL, 0.1 mg/mL, 0.5 mg/mL) to make a standard curve. The quantity of starch hydrolyzing enzyme in the testing samples could be estimated by basing on the standard  $\alpha$ -amylase curve (Farrias *et al.*, 2010). All the tests were triplicated.

### Statistical analysis

The data recorded were subjected to general linear model followed by post-hoc test using SPSS software version 16.0 to compare the means of starch hydrolyzing activities of CFS collected from the cultures in different conditions. The differences were considered as significant at  $p = 0.05$ .

## RESULTS & DISCUSSION

### Identification of starch hydrolyzing bacteria

By biochemical test, bacterium showed positive results with glycerol, L-arabinose, D-xylose, galactose, D-glucose, D-fructose, inositol, mannitol and negative results with erythritol, D-arabinose, L-xylose, adonitol,  $\beta$ -methylxyloside. Under microscope, the bacterium showed gram positive rod with vacuoles, and spore subterminals. By 16S sRNA sequencing analysis, the starch hydrolyzing bacterium isolated in Rach Lang stream in Vietnam showed 99% homology to *Bacillus megaterium* QMB1551, named *Bacillus megaterium* T04 was deposited in DNA Data Bank of Japan (DDBJ) with accession number LC052666.

### Starch hydrolysis ability test

#### Effects of cultivation conditions

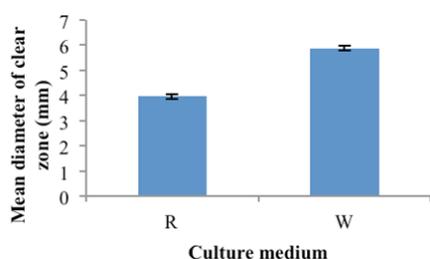
The starch hydrolyzing abilities of the *Bacillus megaterium* T04 were studied in different cultivation conditions including two types of medium (ISP4R and ISP4W) in three levels of cultivation temperature (25, 37, 45°C). Three cultivation periods (24, 48, 72 hours) were studied. The results of this study were shown in table 1.

**Table 1:** Starch hydrolyzing abilities of *Bacillus megaterium* T04 in different conditions.

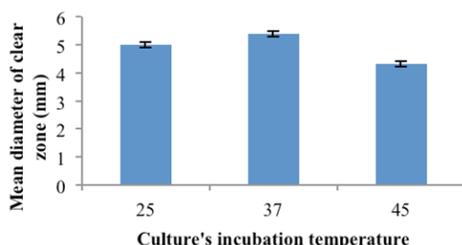
Cultivation condition (medium/ <sup>o</sup> C/hrs)	Diameter of degradation zone (mm)	
	Mean	Standard deviation (SD)
ISP <sub>4R</sub> /25°C/24h	3.2	0.3
ISP <sub>4R</sub> /25°C/48h	4.8	0.6
ISP <sub>4R</sub> /25°C/72h	3.8	0.3
ISP <sub>4R</sub> /37°C/24h	4.8	0.3
ISP <sub>4R</sub> /37°C/48h	1.7	0.3
ISP <sub>4R</sub> /37°C/72h	5.3	0.6
ISP <sub>4R</sub> /45°C/24h	3.8	0.8
ISP <sub>4R</sub> /45°C/48h	3.8	0.6
ISP <sub>4R</sub> /45°C/72h	4.5	0.5
ISP <sub>4W</sub> /25°C/24h	5.8	0.6
ISP <sub>4W</sub> /25°C/48h	6.0	0.5

ISP <sub>4W</sub> /25°C /72h	6.3	0.6
ISP <sub>4W</sub> /37°C /24h	5.8	0.3
ISP <sub>4W</sub> /37°C /48h	8.7	0.3
ISP <sub>4W</sub> /37°C /72h	6.3	0.6
ISP <sub>4W</sub> /45°C /24h	2.5	0.5
ISP <sub>4W</sub> /45°C /48h	6.2	0.6
ISP <sub>4W</sub> /45°C /72h	5.3	0.3

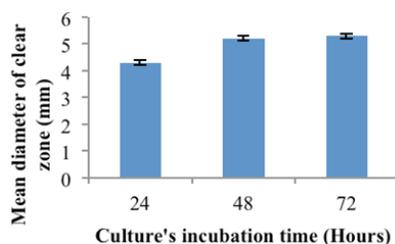
The results revealed that the *Bacillus megaterium* T04 could produce starch hydrolyzing enzyme in all assayed conditions of cultivation. By statistical analysis, there were significant effects of cultivation medium, incubation temperature and incubation time on the production of starch hydrolyzing enzyme of the *Bacillus megaterium* T04. Also, the interactions between these factors were shown significantly in all tests. The activities in all cases were shown in figure 1, 2, and 3.



**Fig. 1:** Illustration for the comparison among different effects of two different media on starch hydrolyzing ability of *Bacillus megaterium* T04. R: ISP<sub>4R</sub> medium, W: ISP<sub>4W</sub> medium.



**Fig. 2:** Illustration for the multiple effects of different cultivation temperatures on starch hydrolyzing ability of *Bacillus megaterium* T04.



**Fig. 3:** Illustration for the multiple effects of different cultivation periods on starch hydrolyzing ability of *Bacillus megaterium* T04.

### Evaluation of ammonium sulfate fractionation

Because the starch hydrolyzing ability was detected in CFSs of *Bacillus megaterium* T04 cultures, the follow-up step fractionation was done to try to recover as much as possible the starch hydrolysis enzymes from the cultures. CFSs from 48-hour cultures at 37°C in ISP<sub>4R</sub> and ISP<sub>4W</sub> were concentrated by ammonium sulfate precipitation at 40% saturation and 60% saturation. The results were shown in the table 2.

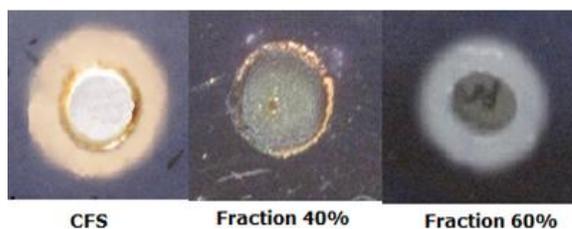
**Table 2:** Diameters of clear zones (mean SD, mm) and their equivalent  $\alpha$ -amylase concentration (mean SD, U/mL) made by *Bacillus megaterium* T04 CFS (cultured at 37°C for 48 hours in ISP<sub>4R</sub>, and ISP<sub>4W</sub> medium) and their ammonium sulfate fractions (40% and 60% saturation).

Culture medium	Sample state	Sample volume (mL)	Clear zone diameter (mm)	Equivalent $\alpha$ -amylase concentration (IU/mL)
ISP <sub>4R</sub>	Crude extract	50	1.7	0.2
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation (40% saturation)	2	0	0
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation (60% saturation)	2	1.8	0.2
ISP <sub>4W</sub>	Crude extract	50	8.7	174.7
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation (40% saturation)	2	0	0
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation (60% saturation)	2	8.8	205.9

There was no enzymatic activity detected in ammonium sulfate fractions at 40% saturation (Table 2, Figure 4, Figure 5). However, the ammonium sulfate fractions at 60% saturation expressed equal or higher enzymatic activity than the crude extracts (Table 2, Figure 4, Figure 5).



**Fig. 4:** Degradation zones produced by *Bacillus megaterium* T04 CFS and ammonium sulfate precipitation fractions of *Bacillus megaterium* T04 cultures in ISP<sub>4R</sub> medium at 37°C for 48 hours.



**Fig. 5:** Degradation zones produced by *Bacillus megaterium* T04 CFS, ammonium sulfate precipitation fractions of *Bacillus megaterium* T04 cultures in ISP<sub>4W</sub> medium, at 37°C, for 48 hours

As seeing in the figure 4 and figure 5, the results of starch agar plate method to detect starch hydrolyzing ability of *Bacillus megaterium* T04 on different kinds of starch such as rice and wheat showed the similar hydrolysis phenomenon after ammonium sulfate precipitation (Figure 4 and Figure 5). *Bacillus megaterium* T04 revealed its starch hydrolyzing ability in all test conditions. Cultivation medium, incubation temperature, incubation time, and their interactions gave significant effects on this capability. It was because they affected to the growth and the metabolisms of the bacteria. Both rice starch and wheat starch in

ISP<sub>4R</sub> and ISP<sub>4W</sub> medium respectively were used as substrate for the growth of *Bacillus megaterium* T04. However, the difference in the composition of rice starch and wheat starch is the reason for the significant difference between the effects of ISP<sub>4R</sub> and ISP<sub>4W</sub>. Rice starch contains more amylopectin, which means that there were more  $\alpha$ -1,6-glycosidic linkages in rice starch than wheat starch. Reversely, more amylose in wheat starch indicates that there are more  $\alpha$ -1,4-glycosidic linkages in ISP<sub>4W</sub> than ISP<sub>4R</sub>. There were different enzymes responsible for starch hydrolysis. They are  $\alpha$ -amylase,  $\beta$ -amylase, glucoamylase, and pullulanase. Among them,  $\alpha$ -amylase and  $\beta$ -amylase only could hydrolyze starch by cutting  $\alpha$ -1,4-glycosidic linkages. Glucoamylase can hydrolyze starch by cutting both  $\alpha$ -1,4-glycosidic linkages and  $\alpha$ -1,6-glycosidic linkages. Nevertheless, pullulanase can cut  $\alpha$ -1,6-glycosidic linkages only (Hii *et al.*, 2012). Interestingly, the enzyme showed 174.7 IU/mL and 0.2 IU/mL in medium containing wheat and rice, respectively. The activities of this enzyme produced by *Bacillus megaterium* T04 were higher than other *Bacillus megaterium* in the previous studies. The study pointed that *Bacillus megaterium* T04 amylase acted strongly in wheat starch that will be potential in pharmaceutical field and other applications, like brewing, environmental areas.

In the effort to recover the starch hydrolyzing enzymes produced by *Bacillus megaterium* T04, fractions of 40% ammonium sulfate didn't present activity, whereas fractions of 60% ammonium sulfate precipitation showed activities. It confirmed the protein nature of the starch hydrolyzing agents produced.

## CONCLUSION

The starch hydrolyzing ability was detected in CFSs from cultures of *Bacillus megaterium* T04 cultured in ISP<sub>4R</sub> and ISP<sub>4W</sub> medium. Both rice starch and wheat starch can be used as the substrates to induce *Bacillus megaterium* T04 to produce starch hydrolyzing enzymes. These biological activities of this strain were found in the cultures up to 72-hour-old and at incubation temperature up to 45°C. These results showed the potentials to produce starch hydrolyzing enzyme in cheap media of ISP<sub>4W</sub>.

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