



## Enzymatic hydrolysis of pangasius belly protein by-product using for *Bacillus subtilis* cultivation

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### ABSTRACT

Through the process of studying the Pangasius belly hydrolysate by neutrase enzyme, the belly Pangasius was defatted; it showed a kinetic index  $V_{max} = 1.283 \mu\text{mol}$  tyrosine per minute,  $K_m = 0.377 \text{ g}$  protein with reliability  $R^2 = 0.997$ , enzyme/substrate ratio (E/S) (0.652 mg enzyme/0.975 g protein). Hydrolysis efficiency of tyrosine according to enzyme/substrate 42.69%, ortho-phthalaldehyde efficiency of the enzyme/substrate was 52.51% at 240 minutes. Subsequently, the belly Pangasius protein hydrolysate was dry-sprayed at 180°C. After drying, the moisture and water activity of dried fish protein hydrolysates were 6.05% and 0.55, respectively. Both commercial peptone and protein hydrolysate from Pangasius belly were used as nitrogen components for *Bacillus subtilis* growth media at the time from 0 to 72 hours at 37°C and pH = 7, then the powder medium hydrolysed by enzyme neutrase was higher than the commercial peptone medium. In addition, the result from the activity of enzyme protease in the two media for at time of 4, 8, 12, 16, 20, 24 hours, at 37°C and pH = 7 showed that the activity of protease in hydrolysed protein medium from the belly Pangasius was similar to commercial peptone.

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## 1 INTRODUCTION

Catfish processing is a big industry, and belly meats obtained from the fillets are considered as by-products. Even though belly meats contain as high protein content as fish muscle, they are currently underutilized as low-valued ingredients. Fish protein hydrolysates (FPH) is used to process hydrolysed protein products by chemical or enzyme (Dufosse *et al.*, 1997). Hydrolysed protein is determined according to various characteristics such as total nitrogen content, soluble nitrogen content, and amino acid composition. The food industry uses FPH exhibiting low hydrolysis rate for their functional such as foaming agents, and low-level hydrolytic emulsifiers for use in the food industry. High hydrolysis of

FPH can be used as a flavour agent after debittering. The medium-level hydrolysed protein used as a microbiological culture medium (Dufosse *et al.*, 1997). Peptones of fish species have been used as a microbiological culture medium, the peptone which is used as a source of protein for fermentation (Annadurai *et al.*, 2012). Peptone was used as a culture medium of *Streptococcus haemolyticus* and *Clostridium botulinum* (Tarr, 1949). Various proteolytic enzymes, such as alcalase, pepsin, papain, flavozyne, and bromelain, have been used in hydrolysis of fish wastes (Kristinsson and Rasco, 2000a, 2000b). However, neutrase, a neutral, metallo-proteinase, requiring zinc ions for its activity, seems to be suitable for hydrolysis of the protein from belly

Pangasius (Rao *et al.*, 1998). In this study, belly Pangasius by-product was hydrolysed and used as nitrogen nutrition for *Bacillus subtilis* growth.

## 2 MATERIALS AND METHODS

### 2.1 Materials

*Bacillus subtilis* bacteria was taken from Institute of Biotechnology Research and Development, Can Tho University. Chemicals such as peptone, acid acetic, casein, folin-ciocalteu's phenol reagent origin from Germany; neutrase enzyme origins from Germany; chemicals such as: disodium hydrogen phosphate dodecahydrate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ), kali dihydrophosphate ( $\text{KH}_2\text{PO}_4$ ), citric acid monohydrate ( $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$ ), trichloroacetic acid 10% (TCA), alcohol 96%, HCl,  $\text{NaHCO}_3$ , sodium hydroxide (NaOH) origin from China and some other chemicals origin from Vietnam. The Pangasius belly must be kept at or below 4°C, regularly iced with the melt water (defrosted ice) free to constantly drain from the fish preservation during 30 minutes transportation (from Can Tho Import – Export Seafood Joint Stock Company (CASEAMEX) to the laboratory of Food Biotechnology department, Can Tho University.

### 2.2 Method

#### 2.2.1 Analytical methods

Protein hydrolysis method by enzyme: Determination of tyrosine content by an improved Anson method (Nguyen Duc Luong, 2003).

Method of lipid content determination: Determining by Soxhlet method (AOAC, 2000).

Total tyrosine content determination: Hydrolysis by acid HCl 6 N (AOAC, 2000)

Acid amine content: Determining amin protein content by OPA method (AOAC, 2000)

Total protein content determination: Kjeldahl method (AOAC, 2000)

Degree of Hydrolysis (DH) = hydrolysed tyrosine/total tyrosine (Nielsen *et al.*, 2001).

Method of growth curve determination (cfu/mL) using Neubauer plate to count bacteria cells (Madigan, 2011).

Protease activity was determined by improved Anson method (Nguyen Duc Luong, 2003)

#### 2.2.2 Experimental set up

Experiment for determine  $V_{\max}$ ,  $K_m$  of enzyme Neutrase on the belly Pangasius

The Pangasius belly was mixed with 10 mL phosphate buffer pH 6.5. Neutrase enzyme was incubated with phosphate buffer at 50°C for 30 minutes, then 0.5 mL enzyme solution was poured into a substrate solution with concentration of 0.02-0.78 g. The duration of hydrolysis was 30 minutes, and absorbance was measured at 660 nm. Protease activity was expressed as tyrosine content released. The  $V_{\max}$  and  $K_m$  of neutrase on the Pangasius belly were determined.

Effects of enzyme and substrate ratio and hydrolysis time on the degree of hydrolysis of hydrolysate from belly Pangasius

In this experiment, the ratios of enzyme/protein (E/S) being 0.5/0.78, 0.625/0.975, 0.75/1.17 and 0.875/1.365 were mixed with 10 mL of phosphate buffer pH = 6.5. The absorbance of the solution was measured at 660 nm (UV-Spectrometer) after different hydrolysis time (0, 30, 60, 90, 120 and 240 minutes, respectively). Hydrolysis efficiency of tyrosine according to enzyme/substrate and orthophthaldialdehyde (OPA) efficiency of the enzyme/substrate was determined.

Protein hydrolysate preparation and growth curve of *B. subtilis* determination

Optimum condition was used to hydrolyse belly Pangasius by-product until getting the highest amine protein content. Then, it was spray-dried at 180°C to be taken for culture of *B. subtilis* in a duration of 0 - 72 hours, temperature 30 °C, pH 7, shake rate 200 rpm. Medium culture was treated at pH = 7 at room temperature. The *B. subtilis* strain was proliferated, diluted and cultured into different nutrient media of  $10^7$  cfu/ mL. Evaluation criteria for growth rate of *B. subtilis* were commercial peptone and the Pangasius belly hydrolysates. In addition, the activity of protease was determined at different time (4 - 24 hours) of culture under an optimum condition (temperature 37 °C, pH =7, shaking rate 200 rpm).

#### 2.2.3 Method of data collection and analysis

The experiment was conducted on the same type of the belly Pangasius getting from the CASEAMEX processing plant, the experiment was repeated twice. The data were processed using SAS 9.1.3 Portable, Stagraphics 15.1 and Excel 2010 software.

## 3 RESULTS AND DISCUSSION

### 3.1 composition of the belly Pangasius

Chemical composition of the belly Pangasius after fat separation shown in Table 1.

**Table 1: Chemical composition of the belly Pangasius**

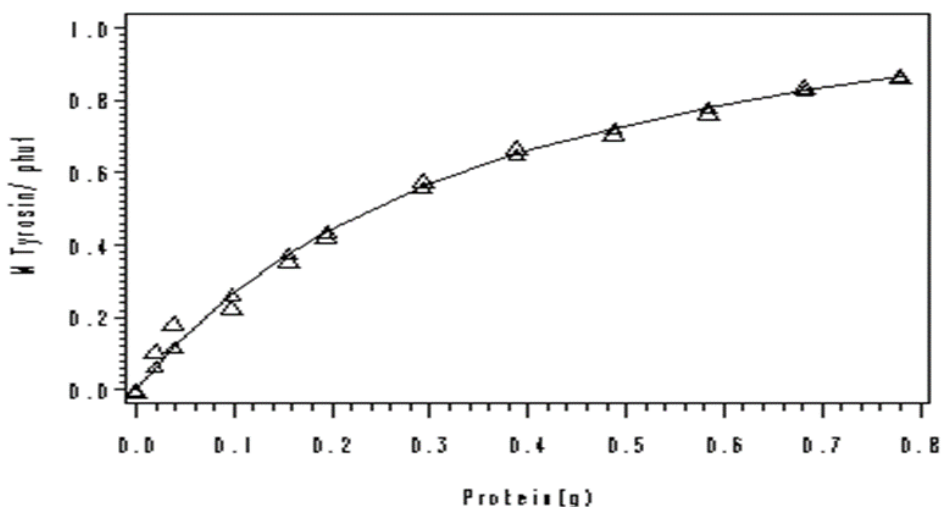
Ingredients	Percentage (%)
Moisture	85±0,5
Lipid	0.74±0.12
Total protein	19,5±0,66

The results showed a relatively high protein content of the Pangasius belly that was a valuable source of by-products with high nutrient value suitable for processing many other products. From the analysis results of the belly Pangasius chemical composition in Table 1, the belly Pangasius has high nutritional value, with total protein content of 19.5%. Compared to the research results of Nguyen Thi My Huong (2011), the total protein content in other parts of yellow fin tuna by-products (*Thunnusalbacares*) such as head, viscera and tail was 14.8, 16.0, and 17.4%, respectively. In addition, Ovissipour *et al.* (2009) also carried out analyzing protein content of total by-products from the beluga fish (*Huso huso*) in a study relating to the process of by-product hydrolysis from this fish. In addition, Ovissipour *et al.* (2009) indicated that the moisture content of visceral by-products of beluga fish was 63.51%; total lipid content was 14.34%, and total protein content

was relatively high at 13.67%. As a result, visceral by-products of beluga fish had a high protein content but were still low in comparison with by-products manufactured from the belly Pangasius. Therefore, the belly Pangasius might create more valuable products.

**3.2 Kinetic parameters of neutrase enzyme on the belly Pangasius**

By content of 0.5 mg (in 0.5 mL phosphate buffer) and gradually increasing the amount of protein from the belly Pangasius (from 0.020 to 0.780 g protein) in a fixed volume of 10 mL phosphate buffer pH 6.5, the study investigated the effect of increasing substrate concentration on the reaction rate of the enzyme neutrase through the amount of tyrosine produced in one minute after hydrolysis. The graph showing the dependent reaction rate of neutrase enzyme on the increasing substrate concentration was drawn by using SAS portable 9.1.3 software (Fig. 1). At the same time, from this software, the kinetic constant  $K_m$  and maximum reaction rate of  $V_{max}$  of neutrase were determined as 0.377 g protein/10ml and 1.283  $\mu$ mol tyrosine per minute with reliability  $R^2 = 0.997$ .



**Fig. 1: The graph showing  $V_{max}$ ,  $K_m$  of neutrase enzyme based on the belly Pangasius**

According to Pagán *et al.* (2013), the effective dissociation constant of neutrase on the protein hydrolysis from the pork bones was 0.036 g protein. Thus, the constant  $K_m$  of neutrase on the Pangasius belly is not much more Pangasius than that on the bone substrate. Large  $K_m$  indicates that the enzyme needs a larger amount of substrate to reach the degree of saturation and reach a maximum reaction rate of  $V_{max}$  (Nguyen Cong Ha *et al.*, 2011). From the ex

periment results, the neutrase enzyme has affinity on protein substrate from the belly Pangasius, showing similarities with the study of Pagán *et al.* (2013). Based on the graph, the neutrase enzyme reached a maximum reaction rate of 0.78 g protein. The volume of enzyme selected for the following experiments is 0.5 mg E/ 0.78 g protein (in 10 mL of phosphate buffer).

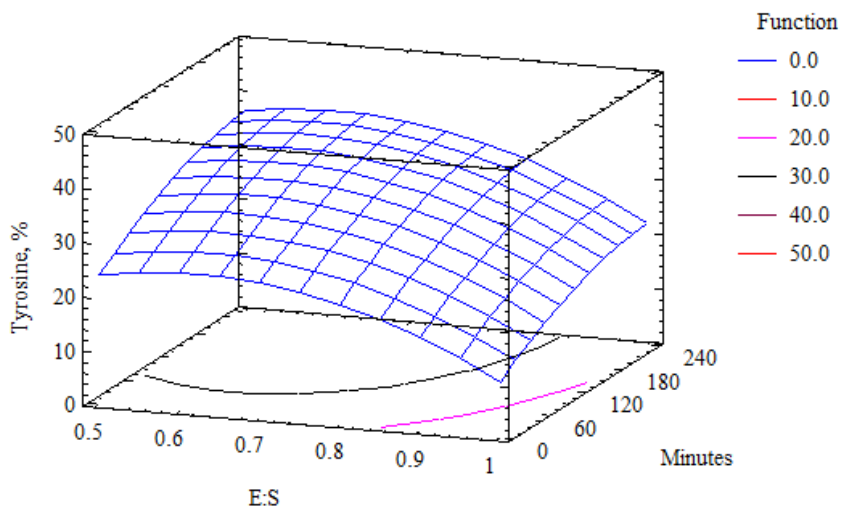
### 3.3 Effects of enzyme/substrate concentration and incubation time on the degree of hydrolysis of hydrolysate from belly Pangasius

#### 3.3.1 Hydrolysis efficiency according to the tyrosine content

Based on Fig. 2, the generated tyrosine content was most efficient at the E/S ratio of 0.625/0.975. The hydrolysis efficiency of neutrase enzyme increases according to the increase of substrate concentration but to a large enough substrate concentration, the neutrase enzyme was inhibited, and the hydrolysis activity of enzyme neutrase was ineffective, at the E/S ratio 0.875/1.365, the hydrolysis efficiency of enzyme starts to decrease despite increased enzyme concentration. The degree of hydrolysis using neutrase enzyme was also gradually increased according to time. This is also consistent with the study of Yao and Zhao (2011) when using neutrase to hydrolyze soybean protein and Pagán *et al.* (2013) when using neutrase to pig bone protein.

Based on the statistical results, the influence of E/S ratio (0.5/0.78, 0.625/0.975, 0.75/1.17, 0.8/1.387)

$$-15.7819 - 0.010949 * X * Y - 105.68 * X * X - 0.00018095 * Y * Y + 0.104009 * Y + 131.88 * X$$



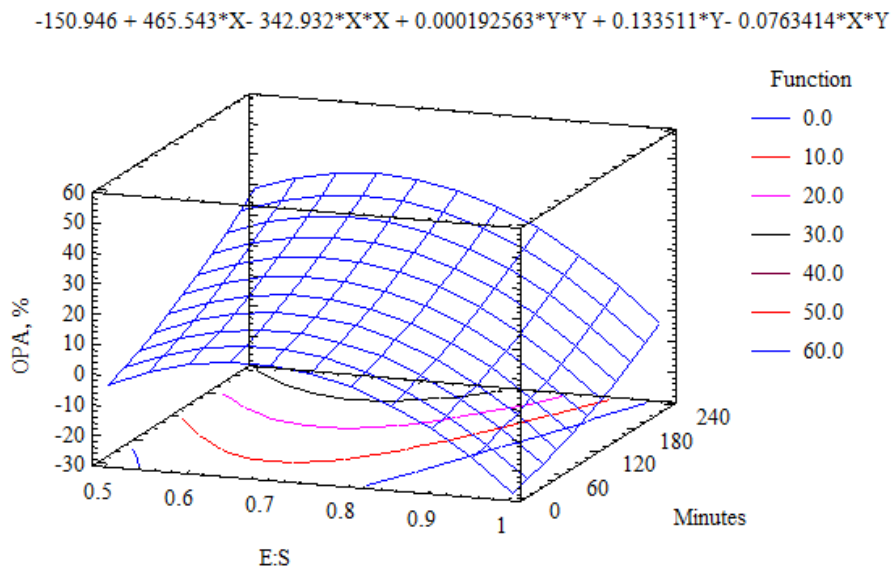
**Fig. 2: Tyrosine hydrolysis efficiency from the belly Pangasius by enzyme neutrase, %**

#### 3.3.2 Hydrolysis efficiency of protein from the belly Pangasius according to amine content

As shown in Fig. 3, the hydrolysis efficiency of neutrase enzyme according to the highest amine content at the E/S ratio (0.625/0.975 mg/g) within 240 minutes was 52.51%. The hydrolysis efficiency of the neutrase enzyme increased with increasing substrate concentration but with a high enough substrate concentration, the enzyme neutrase was inhibited, and the hydrolysis activity of neutrase

and hydrolysis time, after 240 minutes, the equivalent degree of hydrolysis was 34.99%; 42.69%; 34.25%, and 31.52%, respectively. The results on the effect of E/S ratio on the degree of hydrolysis showed that the E/S ratio of 0.625/0.975 (mg/g) was the highest hydrolysis efficiency of 42.69%. The results on the effect of hydrolysis time within 30, 60, 90, 120, 180 and 240 minutes showed that the degree of hydrolysis gradually increased over time, and there was significant difference ( $p < 0.05$ ) among the durations. This degree of hydrolysis is much higher than the related studies. For instance, when using Protamex enzyme to hydrolyse yellow tuna, degree of hydrolysis was just 30.1% at 6 hours (Nguyen Thi My Huong, 2012); when using alcalase enzyme to hydrolyse white sturgeon viscera, the degree of hydrolysis was over 30% at the 120 minutes (Ovissipour, *et al.*, 2009), and when using Alcalase enzyme to hydrolyse cod fish by-products, the degree of hydrolysis was 34.9% (Slizyte *et al.*, 2005). These showed that the hydrolysis of the belly Pangasius by neutrase enzyme is highly effective.

enzyme is ineffective. From the results, at the E/S ratio of 0.875-1.365 (mg/g), the hydrolysis efficiency of enzyme started to decrease despite increased enzyme levels. This experiment result was higher than that of Slizyte *et al.* (2005), the protein hydrolysis efficiency from cod fish by-product by alcalase was 34.9%. The liquid hydrolysed protein was dry-sprayed at 180°C without maltodextrin. The moisture content and water activity of hydrolysed protein powder were 6.05% and 0.55, respectively.



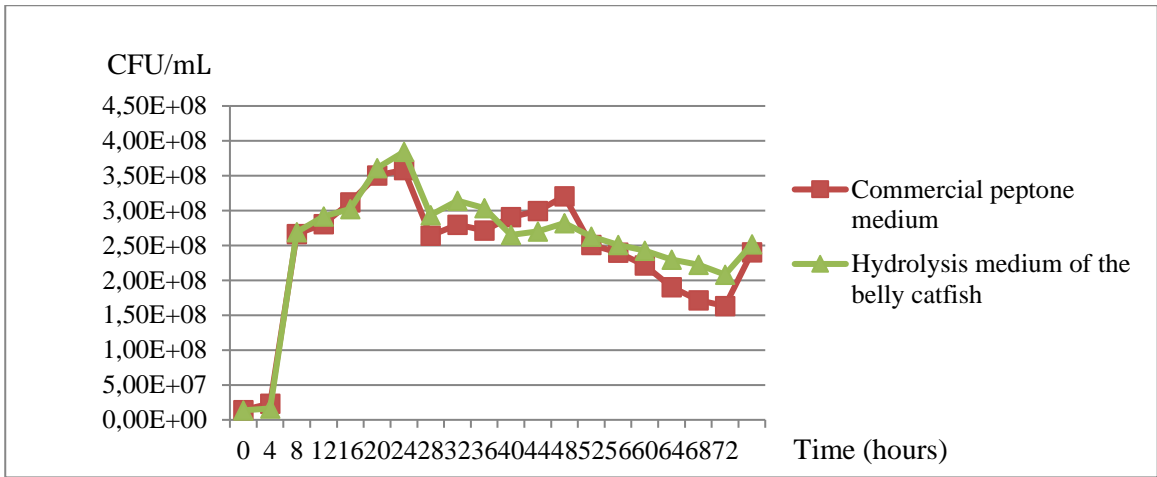
**Fig. 3: Protein hydrolysis efficiency of the belly Pangasius by neutrase enzyme, %**

**3.4 The *Bacillus subtilis* growth in the culture medium containing medium containing hydrolysed protein from belly Pangasius**

According to Fig. 4, *B. subtilis* initial density in culture was  $1.4 * 10^7$  cells/mL. Following the survey time, the density of bacteria gradually increased, and the highest density was reached at 24 hours, for 28 hours. The bacteria density begins stable. After 52 hours, the density decreased gradually at other times. There is a similarity with the study conducted by Truong Thi Nhu Hieu (2010) when culturing of *Bacillus subtilis* on a peptone fish medium, the length of short lag phase was about 0-2 hours, the bacteria started to grow and get maximum cell density at 28 hours. Thus, according to the growth curve of *B. subtilis* bacteria in two types of nutrient mediums of hydrolysed fish by-products, it was noticed that there were two important points affecting the growth and reproduction of *B. subtilis*. Firstly, at 8 hours, this is the time when *B. subtilis* began to grow most strongly and the transition phase from lag

phase to logarithmic phase. The second time is 24 hours when the biomass as well as the cell density in the mediums is highest. At the time of 24 hours in hydrolysed protein from the belly Pangasius by neutrase, the cell density of *B. subtilis* was  $3.84 * 10^8$  CFU/mL, while commercial medium was  $3.58 * 10^8$  CFU/mL. This may be explained that the products from protein hydrolysis of the belly Pangasius are peptides, and *B. subtilis* bacteria amino acid will be easily absorbed. According to Tran Thi Anh Tuyet (2010), the potential growth of *B. subtilis* strain was increased from 6 hours, and the cell density is stable within 18-30 hours.

The statistical results showed a significant difference ( $p < 0.05$ ) of the number of bacteria in the culture media. Thus, for the culture of *B. subtilis* to obtain the density, the culture medium with high cell density should be selected at the optimum and shortest time. From that, a nutrient medium containing the hydrolysed Pangasius belly by neutrase enzymes was chosen for replacement.

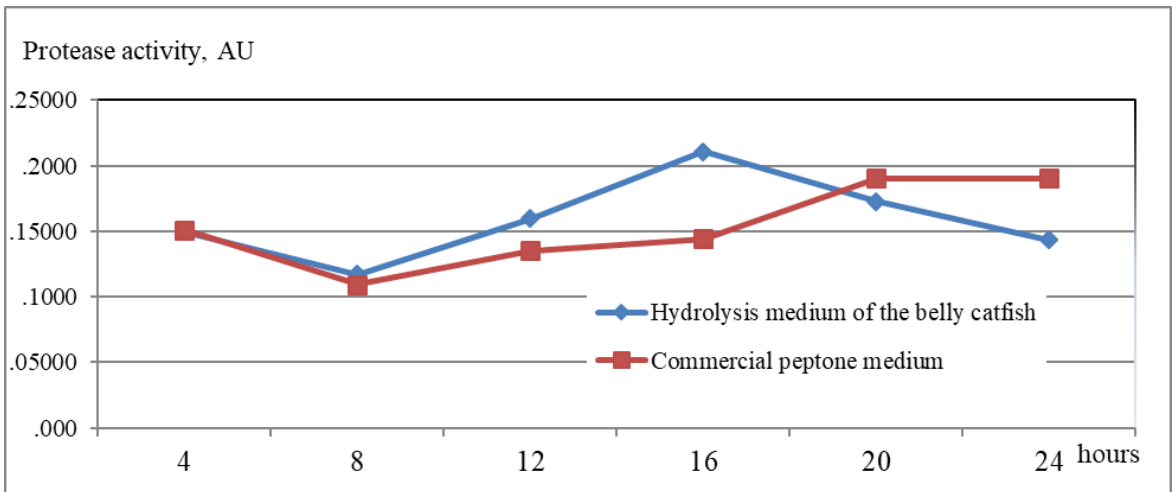


**Fig. 4: Growth curve of *B. subtilis* in nutrient medium of the belly *Pangasius* compared to commercial medium**

**3.5 Evaluation of protease activity on nutrient medium of the *Pangasius* belly**

The medium has a great influence on bacterial protease production. The *Pangasius* belly has many nutrients; therefore, it can be used for bacterial culture. The results from Fig. 5 showed that the *B. subtilis* culture mediums have variable protease activity over time. The protease activity quickly increased from 8 hours to 16 hours of incubation and peaked at the time of 16 hours, the protease activity

achieved 0.211 AU in a medium of hydrolysed fish by-product, and 0.144 AU in commercial peptone medium. Thus, hydrolysis medium of the belly *Pangasius* has a higher protease activity than commercial peptone medium at 16 hours. This result also showed that the maximum time for protease activity was shorter than the study of Truong Thi Nhu Hieu (2010). However, there is no statistically significant difference for the incubation time of two medium, hydrolysed belly *Pangasius* and commercial medium.



**Fig. 5: Protease activity of *B. subtilis* in different nutrient medium**

**4 CONCLUSIONS**

The kinetic parameters of neutrase in belly *Pangasius* substrate were 0.377 g protein and 1.283 μmol tyrosine per minute. The appropriate hydrolysis time of the belly *Pangasius* by Neutrase enzyme was 240 minutes. The degree of hydrolysis was

highest by the E/S ratio (0.625 mg enzyme in 0.975 g protein). Compared to commercial peptone medium, the hydrolysed protein from belly *Pangasius* can be used as a bacterial culture medium based on the development of *B. subtilis* and the activity of the protease formed in the medium.

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