

Production and Determination of Some Biochemical Properties of Protease Enzyme by *Trichothecium roseum* Under Solid State Fermentation

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Abstract

Some fungal species were searched in terms of protease production by using casein degrading protease screening methods and it was found that *Trichothecium roseum*, *Penicillium piceum*, *Penicillium chrysogenum* and *Aspergillus wentii* had the highest protease activity. Solid state fermentation was carried out by using wheat bran as substrate and it was determined that *T. roseum* had the highest protease activity. A number of culture conditions for protease production by *T. roseum* under SSF were investigated. Maximal protease production was obtained with initial moisture content of 85 % (w/v), an inoculum level of 2 mL (1×10^6 spore/mL) when incubated at 30 °C for 7 days. Maximum activity of protease with casein as a substrate was observed after 10 minutes at 40 °C temperature and at pH 6.5. The K_m and V_{max} values of protease for casein as substrate were calculated to be 6.06 (mg/mL) and 121.95 (U/mL), respectively.

Keywords: Protease, solid state fermentation (SSF), *Trichothecium roseum*.

Introduction

Proteases (EC 3.4.21-24 and 99) are enzymes that catalyses hydrolytic reactions in which protein molecules are degraded to peptides and amino acids. These constitute a very large and complex group of enzymes, which differ in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature optima and stability profile [1]. Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. The extracellular proteases are commercial value and find multiple applications in various industrial sectors [2] such as detergent, beer, meat, leather and dairy industries. Thus, proteolytic enzymes are the most important industrial enzymes, representing worldwide sale at about 60% of total enzyme market [3].

Most enzyme manufacturers produced enzymes by submerged fermentation (SmF) techniques. However, in the last decades there has been an increasing trend towards the use of the solid-state fermentation (SSF) technique to produce several enzymes [4]. Solid-state fermentation (SSF) has many advantages including superior volumetric productivity, use of inexpensive substrate, simpler downstream processing, lower energy requirement and low wastewater output [5].

Proteases occur ubiquitously in a wide diversity of sources such as plants, animals, and microorganisms. Microbes are an attractive source of proteases owing to the limited space required for their cultivation and their ready susceptibility to genetic manipulation [6]. Among different types of organisms, fungi are considered more suitable for SSF as their hyphae possess ability to colonize solid substrate and penetrate into inter particle spaces [7]. SSF offers the greatest possibilities when fungi are used because, unlike other microorganisms,

these typically grow in nature on solid substrates [8]. Furthermore, proteases of fungal origin have an advantage over bacterial protease as mycelium can be easily removed by filtration [5].

Some parameters such as fermentation time, temperature, period and initial moisture content, should be optimized to obtain maximum protease production under solid state fermentation.

This work presents, for the first time, studies on a protease produced from wheat bran by *Trichothecium roseum*. Some fermentation parameters were optimized and some properties of protease enzyme were determined.

Material and Methods

Microorganisms and maintenance

Fungal cultures used in this study obtained from Microbiology Research Laboratory Fungus Collection of Trakya University. They were inoculated in test tubes with potato dextrose agar medium (PDA) and incubated at 26 °C for 7 days. Afterwards, the slants were kept at 4 °C until further use.

Inoculum preparation

A volume of 5 mL of sterile 0.9 % NaCl solution was added to fully sporulated 7 days old PDA slant cultures. The spores were scraped by using an inoculation needle under aseptic conditions and obtained homogeneous spore suspension. The spore suspension was diluted and adjusted about 1×10^6 spores/mL and used as the inoculum.

Proteolytic activity screening medium

A qualitative screening medium was used for the proteolytic activity of fungus species. 0.1 % K_2HPO_4 , 0.05 % $MgSO_4 \cdot 7H_2O$, 0.05 % KCl and 1.6 % agar were dissolved in tap water and the mixture was autoclaved for 15 minutes at 121 °C with a pressure of 1.5 atm. Skim milk powder solution which was prepared with distilled water and autoclaved under the same conditions was added aseptically to the mixture and final concentration was adjusted 4.8 % after it was cooled to 60 °C. A volume of 15 mL of the prepared media was transferred to test tubes and they were placed vertically. For each culture 0.1 mL spore suspension was inoculated to tubes and they were incubated at 26 °C for 7 days [9].

Determination of microorganisms which synthesize extracellular protease

The appearance of a clear zone around fungal colony indicates casein hydrolysis. If casein is not hydrolyzed, the media remains white and opaque. The test was evaluated by measuring the depth of clear zone by using a millimeter ruler [9] for 7 days old cultures.

Determination of dry mass of solid substrate

Dry mass of substrate was determined by incubating them in an oven at 80 °C for overnight.

Fermentation medium and culture conditions

Fermentation media containing 5 g of wheat bran (WB) hydrated with distilled water to desired moisture (w/v). The contents were mixed thoroughly and sterilized at 121 °C for 20 minutes in 250 mL Erlenmeyer flasks. The media were inoculated with spore suspension and cultivated at 30 °C for 7 days.

Enzyme extraction

The crude enzyme solution was obtained by adding 50 mL of distilled water to the fermented material. Contents were mixed by shaking for one hour with magnetic shaker at 220 rpm. The slurry was squeezed through muslin cloth. The extract was filtered through a Whatman No.1 filter paper and the filtrate was used as the crude enzyme.

Protease activity assay

Protease activity was determined according to Merheb *et al.* (2007) and Sigma's colorimetric protease activity assay, with a modification [10, 11]. The reaction mixture was made up of 0.4 mL of 0.1 % (w/v) casein solution which is prepared by heating gently to 80-90 °C for 10 minutes with stirring in 50 mM potassium phosphate buffer (pH: 7.5) and 0.4 mL of 50 mM potassium phosphate buffer (pH: 7.5), to which 0.1 mL crude enzyme solution was added. The reaction was carried out at 37 °C for 30 minutes in water bath and stopped by adding 1 mL of 110 mM TCA (trichloroacetic acid). Test tubes were centrifuged at 5000 rpm/5 min and the absorbance of the supernatant was measured at 280 nm. An appropriate control was prepared, in which TCA was added before the enzyme solution. One unit of enzyme activity (U) was arbitrarily defined as the amount of enzyme required to cause an increase of 0.1 in absorbance at 280 nm, under the assay conditions. Enzymatic activity was calculated as follows: $U/ml = (\Delta Abs_{280\text{ nm}} \times 10 \times \text{dilution factor})/0.1$ [10, 11].

Optimization of some parameters controlling protease production on SSF

Various process parameters influencing enzyme production during SSF were optimized. The strategy followed was to optimize each parameter, independent of the others and subsequently optimal conditions were employed in all experiments [12].

The effect of production period was determined by incubating SSF medium for different incubation periods viz.: 3, 4, 5, 6, 7, 8, 9 and 10 days with 1 mL inoculum concentration, 50 % (v/w) moisture at 30 °C, respectively. The effect of production temperature was performed by incubating at different incubation temperatures viz.: 20, 30, 40 and 50 °C for 7 days. The spore suspension was prepared as previously mentioned and different inoculum sizes were applied viz.: 1.0, 1.5, 2.0, 2.5 and 3.0 mL. The initial moisture content of SSF medium was optimized by adding 45, 55, 65, 75, 85 and 95 % (w/v) distilled water.

Some biochemical properties of protease enzyme

In the present study, crude enzyme extracts were used to investigate some properties of protease enzyme. Optimal incubation temperature was determined by incubating protease enzyme at different temperatures viz.: 20, 30, 40, 50 and 60 °C, for 30 minutes in 50 mM potassium phosphate buffer (pH: 7.5), respectively. The effect of pH was measured in pH 6.0, 6.5, 7.0 and 7.5 potassium phosphate buffers and pH 8.0 borate buffer at 40 °C for 30 minutes. The effect of incubation period on protease activity was determined by incubating at different periods viz.: 5, 10, 20, 30 and 40 minutes in potassium phosphate buffer (pH: 6.5) at 40 °C. Casein solutions at different concentrations (1, 2, 3, 4, 5 and 6 mg/mL) were prepared to obtain K_m value of protease enzyme. The reactions were performed at 40 °C for 10 minutes and protease activities were measured for each substrate concentrations. $1/[S]$ and $1/[V]$ values were calculated and Lineweaver-Burk chart was drawn. K_m and V_{max} values were determined by using this chart.

Results and Discussion

Screening of extracellular protease producers

In this study, 47 fungus cultures were searched in terms of extracellular protease enzyme activity. Four cultures which synthesized extracellular protease enzyme and hydrolyzed casein in the protease screening medium more than the others were determined as *Trichothecium roseum*, *Aspergillus wentii*, *Penicillium piceum* and *Penicillium chrysogenum* (Table 1.).

Table 1. Microorganisms which synthesize extracellular protease

| Microorganisms | Clear zone (mm) | Microorganisms | Clear zone (mm) |
|-------------------------------------|-----------------|-----------------------------------|-----------------|
| <i>Alternaria alternata</i> | 5 | <i>Penicillium crustosum</i> | 12 |
| <i>Aspergillus ademetzii</i> | 5 | <i>Penicillium donkii</i> | 10 |
| <i>Aspergillus alliaceus</i> | 18 | <i>Penicillium expansum</i> | 13 |
| <i>Aspergillus expansum</i> | 8 | <i>Penicillium funiculosum</i> | 4 |
| <i>Aspergillus fumigatus</i> | 11 | <i>Penicillium glabrum</i> | 20 |
| <i>Aspergillus granulatum</i> | 14 | <i>Penicillium griseofulvum</i> | 21 |
| <i>Aspergillus niger</i> | 20 | <i>Penicillium griseoroseum</i> | 15 |
| <i>Aspergillus terreus</i> | 14 | <i>Penicillium herquei</i> | 14 |
| <i>Aspergillus wentii</i> | 24 | <i>Penicillium hirsulum</i> | 13 |
| <i>Cladosporium cladosporioides</i> | 16 | <i>Penicillium jensei</i> | 20 |
| <i>Cladosporium cucumerinum</i> | 17 | <i>Penicillium miczynskii</i> | 16 |
| <i>Cladosporium herbarum</i> | 10 | <i>Penicillium piceum</i> | 25 |
| <i>Cladosporium sphaerospermum</i> | 11 | <i>Penicillium roquofortii</i> | 9 |
| <i>Cladosporium tenuissimum</i> | 8 | <i>Penicillium simplicissimum</i> | 20 |
| <i>Drechslera bicolor</i> | 20 | <i>Penicillium viridicatum</i> | 22 |
| <i>Drechslera hawaiiensis</i> | 10 | <i>Penicillium waksmanii</i> | 20 |
| <i>Mucor</i> sp. | 8 | <i>Penicillium glabrum</i> | 16 |
| <i>Penicillium aurantiogriseum</i> | 8 | <i>Rhizopus</i> sp. | 10 |
| <i>Penicillium bilaai</i> | 15 | <i>Scopuloriopsis brevicaulis</i> | 20 |
| <i>Penicillium brevicompactum</i> | 19 | <i>Trichoderma</i> sp. | 12 |
| <i>Penicillium camemberti</i> | 12 | <i>Trichothecium roseum</i> | 23 |
| <i>Penicillium chrysogenum</i> | 26 | <i>Ulocladium alternaria</i> | 16 |
| <i>Penicillium citrinum</i> | 18 | <i>Ulocladium atrum</i> | 9 |
| <i>Penicillium corylophyllum</i> | 17 | | |

Trichothecium roseum, *Aspergillus wentii*, *Penicillium piceum* and *Penicillium chrysogenum* were used for protease production on SSF medium. After the protease activity assays, *T. roseum* was obtained the best protease producer and the most convenient culture for SSF and *T. roseum* was chosen as the protease source for further studies. The relative protease activities were 97.22 % for *Penicillium chrysogenum*, 50 % for *Penicillium piceum* and 38.88 % for *Aspergillus wentii* (Table 2.).

In order to improve the protease production, further parameters were investigated.

Table 2. Relative protease activities of fungal cultures grown in SSF

| Microorganisms | Relative Activity (%) |
|--------------------------------|-----------------------|
| <i>Trichothecium roseum</i> | 100 |
| <i>Penicillium chrysogenum</i> | 97.22 |
| <i>Penicillium piceum</i> | 50 |
| <i>Aspergillus wentii</i> | 38.88 |

Optimization of some parameters controlling protease production on SSF

Any fermentation process is governed by a large number of physical, chemical and biological factors [13]. However, some parameters highly affect enzyme production by SSF such as fermentation time, fermentation temperature, inoculum size and initial moisture content.

Fermentation period

The culture of *Trichothecium roseum* is effectively colonized the surfaces of wheat bran in solid state fermentation and exhibited dense growth. The enzyme production was gradually increased with the passage of time and the highest protease activity was obtained after seven days incubation. The enzyme activity has decreased after seven days (Figure 1.). It was reported that *Aspergillus niger* [14], *Aspergillus* sp. [15], *Aspergillus flavus* and *Aspergillus terreus* [16] had maximum protease activity on 3rd day of incubation. Sindhu *et al.* (2009) indicated that *Penicillium godlewskii* produced maximum protease enzyme after four days incubation [17]. Lazim *et al.*, (2009) reported that maximum protease activity was obtained from *Streptomyces* sp. CN902 on 5 days incubation [18].

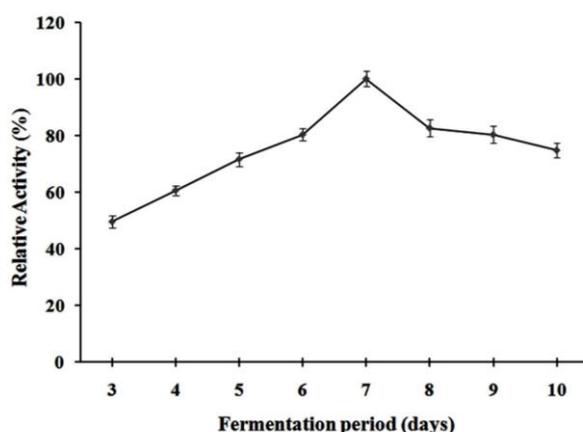


Figure 1. Effect of fermentation period on protease production. Fermentation conditions: temperature of fermentation 30 °C; inoculum size 1 mL; initial moisture 50 % (v/w).

Fermentation temperature

Medium temperature plays an important role in the protease production [19]. Thus, SSF medium was incubated various temperatures (20-50 °C) to determine optimal temperature for *Trichothecium roseum* protease. The results indicated that *T. roseum* highly produces protease at 20 °C and 30 °C. As showed in Figure 2, optimum fermentation temperature was 30 °C for *T. roseum*. The protease production suddenly decreased when the incubation temperature was 40 °C and 50 °C. Higher temperature is found to have some adverse effects on metabolic activities on microorganism [20] and cause inhibition of the growth of fungus. The enzyme is denatured by losing its catalytic properties at high temperature due to stretching and breaking of weak hydrogen bonds within enzyme structure [21]. Additionally, high temperature would accelerate the volatilization of water in the medium, thus restraining the growth and enzymatic production of the microbe [22].

Similarly, optimum fermentation temperatures were reported as 30 °C for *Rhizopus microspores* [23], *Penicillium chrysogenum* [24], *P. citrinum*, *P. purpurogerum* and *P. funiculosum* [25]. Furthermore, it was reported that *Aspergillus niger* [14] and *Streptomyces* sp. CN902 [18] had maximum protease activity at 35 °C and 45 °C, respectively.

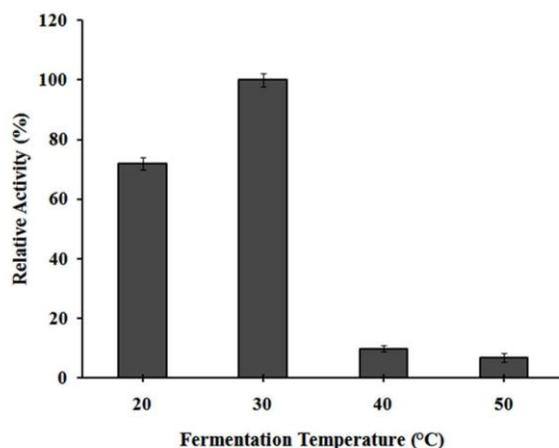


Figure 2. Effect of fermentation temperature on protease production. Fermentation conditions: period of fermentation 7 days; inoculum size 1 mL; initial moisture 50 % (v/w).

Inoculum size

Trichothecium roseum showed highest protease production when 2 mL spore suspension (1×10^6 spore/mL) was inoculated to SSF medium. The relative protease activity was 97.13 % when 1,5 mL spore suspension was used. However, the protease production has decreased when the amount of inoculum was below 1,5 mL and above 2 mL (Figure 3.). These results indicate that highest inoculum amounts cause a decrease of enzyme production.

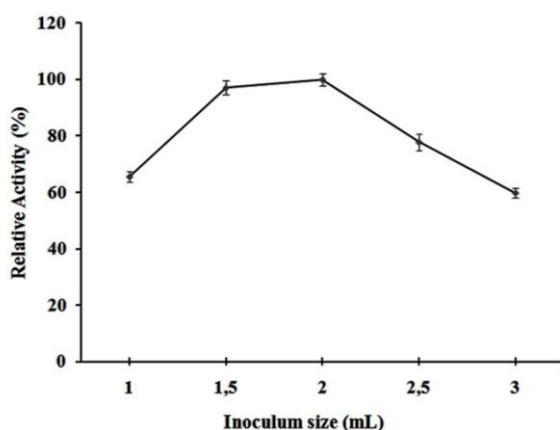


Figure 3. Effect of inoculum size on protease production. Fermentation conditions: period of fermentation 7 days; temperature of fermentation 30 °C; initial moisture 50 % (v/w).

Initial moisture content

The moisture level must both supply water for fungal growth and facilitate the colonization of fungus on solid substrate. It also must provide heat transmission. Therefore, optimization of initial moisture is pretty important. Optimum moisture content for the production of protease by *T. roseum* was 85 % (Figure 4.). The initial moisture contents had reported as 50 % for *Penicillium* sp. [26], 55 % for *Penicillium* LPB-9 [27], 63 % for *Aspergillus flavus* IMI 327634 [28] and 140 % for *Rhizopus oryzae* [29] on previous studies.

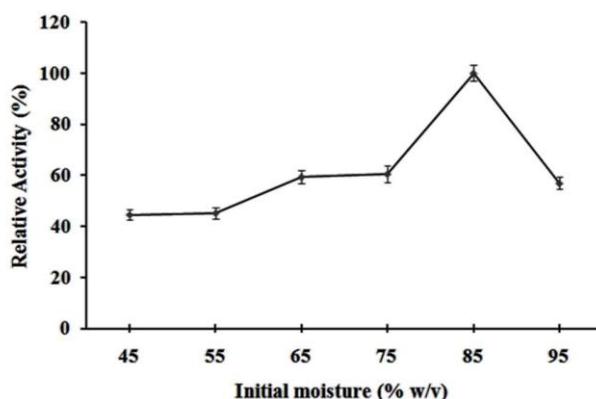


Figure 4. Effect of initial moisture on protease production. Fermentation conditions: period of fermentation 7 days; temperature of fermentation 30 °C; inoculum size 2 mL.

Some biochemical properties of protease enzyme

Effect of incubation temperature

Temperature is a critical factor for maximum enzyme activity and it is a prerequisite for industrial enzymes to be active and stable at higher temperature [30]. *T. roseum* indicated maximum protease activity at 40 °C. However, the enzyme was not completely inactivated at 60 °C because it had 49.35 % relative activity at that temperature (Figure 5.). The optimum incubation temperatures were reported as 45 °C for *Aspergillus niger* alkaline protease [30], 50 °C for *Thermomyces lanuginose* P₁₃₄ alkaline protease [31], 35 °C for *A. nidulans* alkaline protease [32], 45-60 °C for *A. terreus* and *A. flavus* alkaline proteases [16].

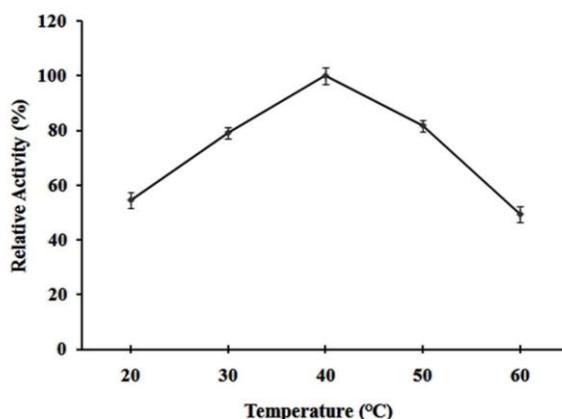


Figure 5. Effect of incubation temperature on protease activity. Incubation conditions: pH: 7.5; 30 min.; substrate concentration 0,4 mg/mL.

Effect of incubation pH

The protease activity increased slowly from pH 6.0 and reached maximal rate of reaction at pH 6.5. However, the relative protease activities were 87.04 and 77.78 % at pH 7.0 and 7.5, respectively (Figure 6.). Thus, the enzyme appeared to be slightly acidic and neutral. Fungal neutral proteases are the most important component of commercial fungal protease preparations, which have applications in baking, food processing, protein modification, and in the leather, animal feeds and pharmaceutical industries [33].

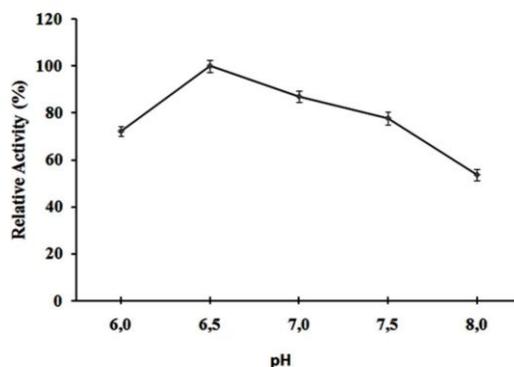


Figure 6. Effect of incubation pH on protease activity. Incubation conditions: temperature 40 °C; 30 min.; substrate concentration 0,4 mg/mL.

Effect of incubation period

As the enzyme –substrate interaction period increase, the amount of products also increase up to a certain point. Thus, understanding of the reaction time might assist to know when the maximum products occurred [34]. The maximum level of *Trichothecium roseum* protease activity was obtained after 10 minutes incubation when the reaction was carried out at 40 °C. However, it maintained 70, 64 and 60 % of its activity after 20, 30 and 40 minutes incubation, respectively (Figure 7.).

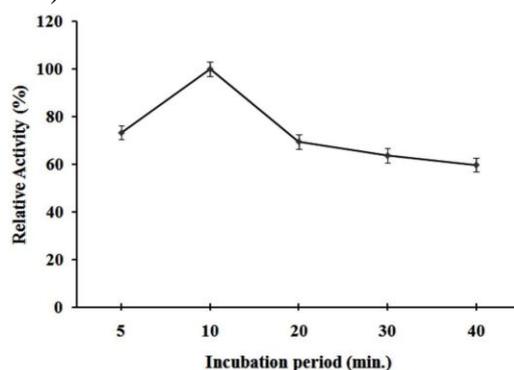


Figure 7. Effect of incubation period on protease activity. Incubation conditions: temperature 40 °C; pH: 6.5; substrate concentration 0,4 mg/mL.

Determination of K_m and V_{max} values

Optimum substrate concentration for maximum protease activity was determined in terms of V_{max} and K_m using casein as a substrate. V_{max} and K_m values were interpreted from Lineweaver-Burk Plot. Kinetic parameters of the protease in crude extract revealed that the V_{max} and K_m values were 121.95 U/mL and 6.06 mg/ml, respectively (Figure 8).

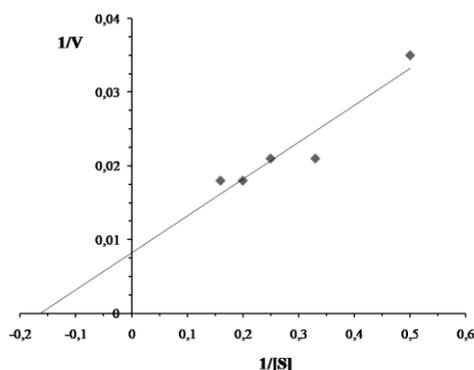


Figure 8. Lineweaver-Burk Plot. Incubation conditions: temperature 40 °C; pH: 6.5; 10 minutes.

Conclusions

In this study, protease enzyme of *T. roseum* was produced by SSF which is economical and successful method for enzyme production. Highest protease activity was obtained on 7th day of fermentation at 30 °C with 85 % initial moisture and an inoculum level of 2 mL (1×10^6 spore/mL). Some biochemical properties of crude enzyme were determined and it was found that the enzyme had maximum activity at 40°C and pH 6.5 when incubated for 10 minutes. The enzyme had 6.06 mg/mL K_m value and was not isolated from *T. roseum* before. On the light of the biochemical properties of *T. roseum* protease which were obtained in this study, *T. roseum* could be suggested as a new protease producer under solid state fermentation for industrial applications. The suitability of *T. roseum* to SSF will provide advantage for industrial applications.

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