In vitro, determination of optimal conditions of growth and proteolytic activity of clinical isolates of *Trichophyton rubrum*

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Objective To determine the effect of some growth conditions in proteolytic activity of clinical isolates of *Trichophyton rubrum*.
Methods Isolation and identification of a dermatophyte *T. rubrum* from hair and skin scrapings of patients with dermatophytosis by using the morphological and cultural characteristics. Optimal growth conditions of eight isolates including temperature, pH, culture media type and incubation period were studied, in addition to that the proteolytic activity and its optimal production in liquid media were tested.
Results The results showed that the colonies of *T. rubrum* on Sabouraud dextrose agar (SDA) were like cotton or powder-like, white or light beige, flat or elevated, with or without pigments on the reverse. The optimal conditions of growth were 30°C, pH6 and SDA media. The proteolytic activity against casein as substrate showed that *T. rubrum* isolates have an ability to produce exocellular protease ranged from 10.5–80.1U/ml according to the source of each isolates. On the other hand, the proteolytic activity varied based on the pH value, temperature, incubation period and concentration of substrate.
Conclusion The present data may refer to the vital role of proteolytic activity in the invasion and pathogenesis of *T. rubrum* infection.
Keywords *Trichophyton rubrum*, growth conditions, proteolytic activity.

Introduction

The dermatophytoses are among the most common human disease. Although the prevalence of those infections varies greatly, at least 10–20% of the world population may be infected with dermatophytes.[1]

The highly developed host-parasite relationship is responsible for a multitude of clinical manifestation and many of these host-parasite interactions depend upon specific moieties and enzyme production, especially in *Trichophyton rubrum* which may enhance survival in tissues by chemically or physically altering the immediate environment or they may act directly by digesting host proteins, thus providing a source of nutrition.

More attention has been given to the enzymes produced by pathogenic fungi due to their role in human pathogenicity. However, exoenzymes are found to be produced by dermatophtes are keratinases, lipases, phospholipases, elastases, collagenases and proteases.[2]

The dermatophytes are a group of closely related fungi that have the capacity to invade keratinized tissue (skin, hair, nail, feathers, horns and hooves) of human and other animals to produce an infection, dermatophytosis. About 40 species belonging to the genera *Microsporum*, *Trichophyton* and *Epidermophyton* are considered as dermatophytes. They possess two important properties, keratinophilic and keratinolytic. This mean that they have the ability to digest keratin *in vitro* in their saprophytic state and utilise it as a substrate, and some may invade tissues *in vivo* and provoke tineas.[3]

T. rubrum is the most common causative agent of dermatophytosis worldwide, mainly occupying the humans' feet, skin and between fingernails. *T. rubrum* is known to be one of the most prominent anthrophilic species of dermatophtyes, a fungus commonly causing skin diseases.[4] Very little is known about the mechanism of its invasion and pathogenicity.[5] Though it is usually not life-threatening, infections are long-lasting, recurring and incredibly difficult to cure. The fungal pathogen's ability to produce and secrete proteolytic enzymes is a major virulence factor.[6] The aim of the present study is the isolation and identification of *T. rubrum* from clinical specimens with dermatophytosis by conventional method (macroscopic and microscopic characteristics, biochemical and physiological tests, testing the optimal temperature, pH, culture media, substrate concentration and incubation period for growth and proteolytic activity of protease produced by *T. rubrum* isolates *in vitro*).

Materials and Methods

Clinical Specimens

A total of 150 clinical specimens (hair, nails and skin scrapings) were collected from patients who attended the dermatology and venereal disease centre at Mergan hospital and private clinic in Babylon city from February 2014 to May 2014. The specimens were inoculated on Sabouraud's dextrose agar (SDA) containing cycloheximide (0.5 g/l) and chloramphenicol (0.05 g/l) at pH 5.6 and incubated at 29±2°C for 14–21 days. Method of isolation and identification of fungi were performed as previously published.[7–9]

Influence of Environmental Factors in the Growth of *T. rubrum*

1. Temperature

To examine the effect of temperature on growth of eight isolates of *T. rubrum*, preliminary experiments were investigated using temperature which ranged from 20°C to 40°C. Then the following temperature regimes i.e. 20°C, 25°C, 30°C, 35°C and 40°C were conducted by taking a disk (0.5 cm) of fungal tissues from the edge of colony age (7–10 days) using cork borer. The centre of new petri dishes containing SDA medium were inoculated using three replicates. Fungal growth of *T. rubrum* were calculated by taking two intersecting lines from the

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centre of the dish at 2 days interval for 7 days.[10]

2. pH

To examine the effect of pH on growth of eight isolates of *T. rubrum*, the following pH regimes i.e. 5, 6, 7 and 8 were conducted. All the other steps like inoculation technique, incubation time, replication and growth measurements for the fungus were applied as previously mentioned; except that the incubation temperature was 30°C.[11]

3. Culture media

The effect of four types of media i.e. SDA, PDA, CMA, and YEA on growth of eight isolates of *T. rubrum* were investigated, using similar steps that were applied as previously mentioned; except the incubation temperature was 30°C and the pH of growth medium was 6, replication and growth measurements were done as previously mentioned.[12]

Protease Production Media

The cells of *T. rubrum* isolates were grown on synthetic medium composed of peptone (10 g/L) and dextrose (40 g/L) with pH 5.4, and was autoclaved at 121°C for 15 min, cooled in flasks (250 ml size), and inoculated by placing 0.5 cm agar medium plugs containing active mycelium (5 days old) from the fungus growing in slant culture. Flasks were incubated at 35°C on a rotary shaker at 150 rpm for 30 days.[13]

Determination of Protease Activity

Proteolytic activity against casein substrate was measured according to the method published by Hanlon and Hodges (1981).[14] The reaction mixture contained 0.5 ml of substrate, 0.5 ml of culture filtrate and 0.1 ml of 0.5M tris-HCl buffer, pH8. The reaction was carried out at 40°C for 30 min, stopped by addition of 2 ml of 0.67M trichloroacetic acid (TCA) and then allowed to stand for 1 h. The precipitate was removed by centrifugation at 3000 g for 15 min. Absorbance of the supernatant was measured at 280 nm by spectrophotometer against a reaction blank prepared as above except that TCA solution was added before the addition of the culture filtrate. One unit of enzyme activity was defined as the amount of enzyme that could liberate products having an absorbance of 0.1 under described conditions. The specific activity was expressed as the number of units of activity per mg protein.

Absorbance was used to calculate the activity of enzyme using the formula:

$$\text{Enzyme activity U/ml} = \frac{(\text{O.D})}{0.01 \times \text{Time} \times \text{volume (crude enzyme)}}$$

Effect of some Culture Conditions on the Production of Protease

In this work, the activities of protease were measured under several nutritional conditions including substrate concentration (0.5, 1, 1.5, 2, 2.5, 3%), incubation intervals (3, 6, 9, 12, 15 days), pH of culture media (5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9) and the incubation temperature (20, 25, 30, 35, 40, 45, 50˚C).[15]

Statistical Analysis

Statistical analysis was performed by using SPSS computing program for the analysis of the results. A P-value under 0.05 was considered statistically significant.[16]

Results

Influence of Environmental Factors on the Growth of T. rubrum Isolates

1. Temperature

The results of effect of different temperature (20, 25, 30, 35, 40°C) in growth rate of eight isolates of *T. rubrum* grown in SDA during different periods of incubation (2, 5, 7 days) revealed significant differences ($P \leq 0.05$) and 30°C was the optimal temperature at the seventh day of incubation for all studied fungal isolates, where the colonies diameter of isolates No. 1 to No. 8 were (3.5, 3.4, 3.7, 4, 9, 3.7, 7.3 and 6.5 cm) respectively, (Fig. 1).

2. pH

Figure 2 shows the results of testing eight isolates of *T. rubrum* for growth under different pH values (5–8) that were grown on SDA at 30°C and different incubation periods (2, 5, 7 days) showed that the pH = 6 was the optimal pH value for growth of all tested isolates of *T. rubrum* after 7 days of incubation and the statistical analysis showed a significance difference ($P \leq 0.05$) among tested treatments. On the other hand, the growth of colonies diameter of fungal isolates (No. 1 to No. 8) were (4.2, 4, 4.5, 3.9, 9, 3.7, 7.3 and 6.5 cm) respectively.

3. Culture media

The data of effect of culture media (SDA, PDA, CMA, YEA) in growth rate of eight isolates of *T. rubrum* during different periods of incubation (2, 5, 7 days) and 30°C is shown in Fig. 3, the optimal growth for all tested isolates (No. 1–8) were (4, 4.5, 4.3, 4.7, 9, 4.3, 7.3 and 6.5 cm), respectively, on SDA rather than other used media and after 7 days of incubation. A statistical analysis showed a significance difference ($P \leq 0.05$) among tested treatments.

Proteolytic Activity of T. rubrum Isolates

Results of present study showed that *T. rubrum* isolates have the ability to produce exocellular protease which was indicated by proteolytic activity against casein when added as substrate to the culture filtrates of *T. rubrum*. After 9 days of growth, the *T. rubrum* No. 1 showed the highest proteolytic activity (80.1 U/ml) and the minimum activity was showed by the *T. rubrum* No. 8 (10.5 U/ml).

The influence of cultural conditions (substrate concentration, pH, incubation period and temperature) on the production of protease by *T. rubrum* isolates were investigated. The *T. rubrum* isolates showed high ability to produce protease 48.3 U/ml at 0.5% (Fig. 4). Also the result showed that high activity of the enzyme obtained at the 9th day of incubation, reached to 88.5 U/ml, and then a progressive decrease in proteolytic activity was observed when incubated for 12 and 15 days, as shown in Fig. 5.

On the other hand, results showed that the proteolytic activities of *T. rubrum* isolates were rapidly increased, reaching a maximum of 78.5 U/ml at pH7, then a progressive decrease in protease production occurred with increasing pH values (Fig. 6). The highest proteolytic activity 83.6 U/ml achieved by *T. rubrum* isolates when incubated at 30°C, then decreased with the increase of temperature, while the isolates lost its ability to produce proteolytic activity when incubated at 50°C (Fig. 7).

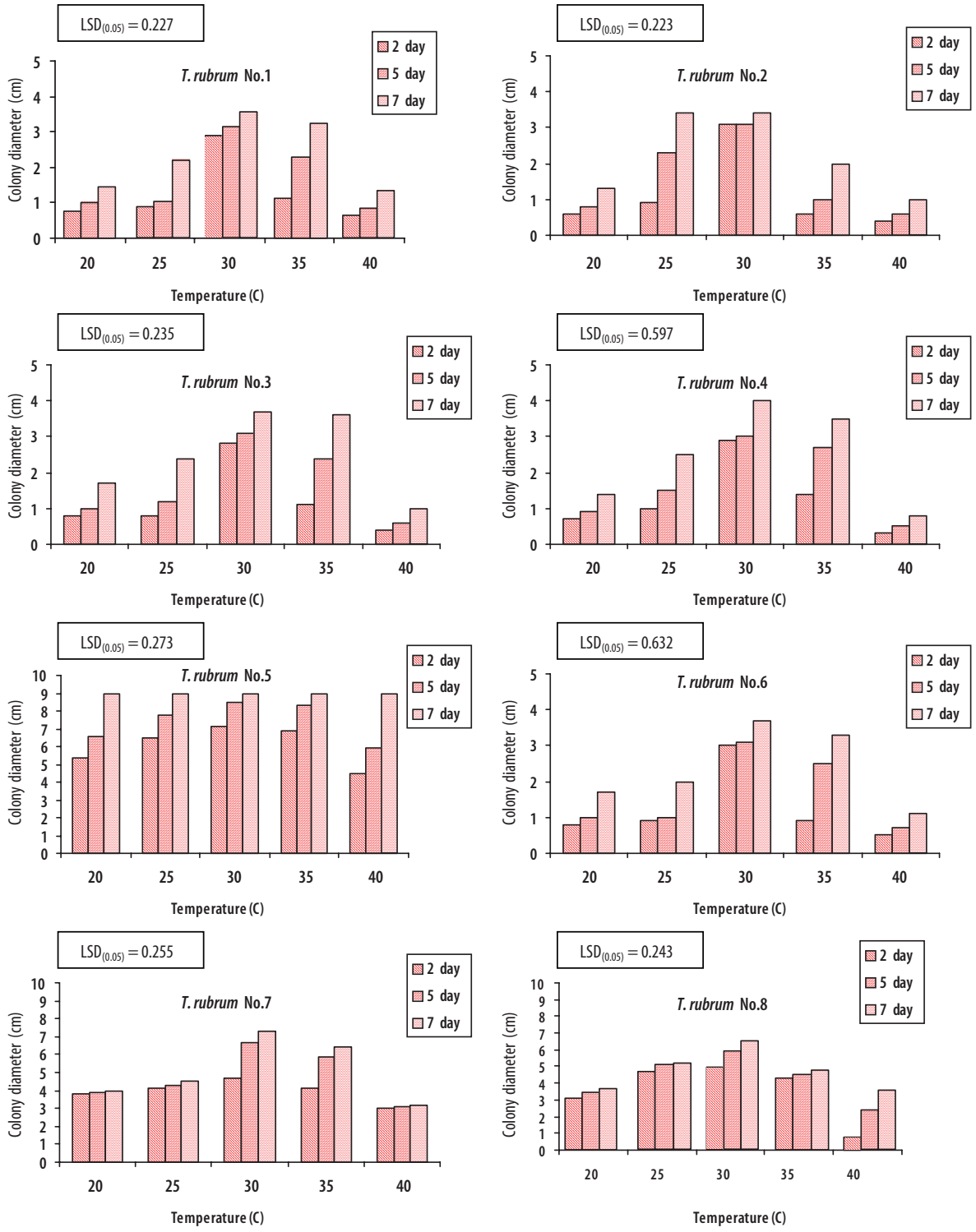

Fig. 1 **Effect of different temperature on the growth of *T. rubrum* isolates on SDA after 7 days of incubation.**

Discussion

Environmental factors play an important role in the growth of keratinophilic fungi. Temperature is one of the important ecological factors that affects the growth of microorganisms and reproduction, every fungus has a definite range of temperature within which it grows and sporulates. Usually most of the fungi grow at temperature ranging from 15°C to 35°C; some of the fungi require a range of higher temperature for their optimum growth.[17,18] All isolates of *T. rubrum* grew well at temperature between 25°C and 35°C but the maximum growth varied according to the isolate type. It was found that 30°C and 35°C were the most suitable for optimum growth of most of the isolates during 7 days in SDA medium in the pH = 6, but colony diameters were reduced in low temperature (2°C) and high temperature (40°C) which may inhibit the growth of fungi compared with other temperatures (25, 30, 35°C).

The present results are in agreement with Sharma et al.,[19] who found that 33°C was most suitable for *T. rubrum*

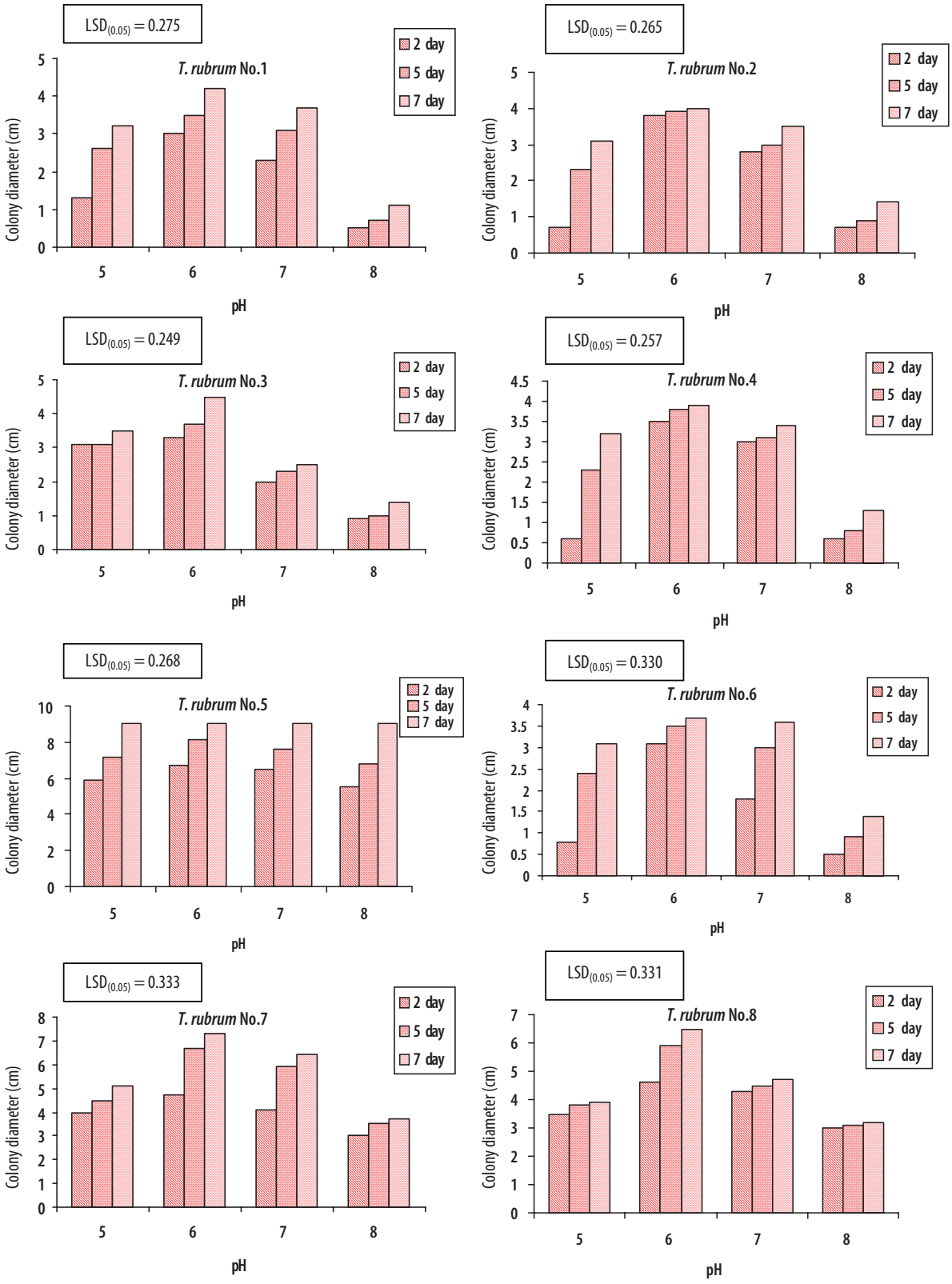

Fig. 2 **Effect of different pH on the growth of *T. rubrum* isolates after 7 days of incubation.**

in terms of dry weight of mycelium as well as in colony diameter.

High temperature could lead to fungal cell rupture and loss of membrane or damage to the intra-cytoplasmic compounds and cell analyses where De Maranon et al.,[20] found that maximum growth was obtained at 32°C because the dermatophytes grow best in culture at temperature lower than human body. Increasing or decreasing of temperature than optimum range may lead to the breakdown of enzymes.[21] So increase in the rate of growth at 29–35°C may attribute to enzyme activity which reaches to the top at optimum temperature and then they use the food source in the media to build macro-molecular and then build the fungal mass. In addition, respiration process gets reduced at low temperature

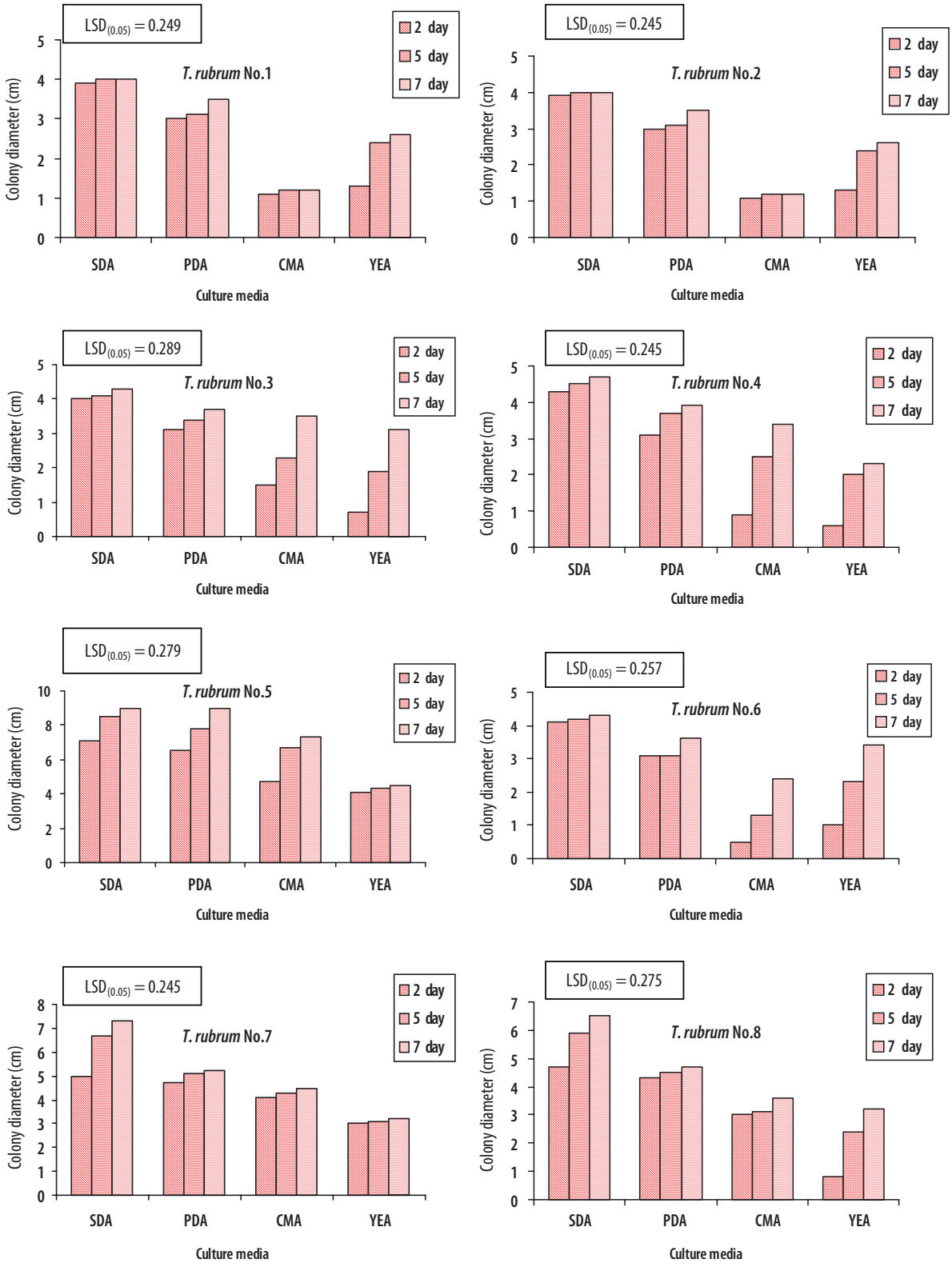

Fig. 3 **Effect of different culture media on the growth of *T. rubrum* isolates after 7 days of incubation.**

but at higher temperature the process will stop which leads to the death of the fungi.[21]

Dermatophytes could grow over a wide range of pH. This result demonstrated the important role of pH in the growth of *T. rubrum* isolates in different pH (5, 6, 7 & 8) in SDA media for 7 days at 30°C. It was found that pH 6 was the most suitable for fugal growth.

Fungi can tolerate a wide range of pH and change the pH as they grow, some species increase the pH of medium but others decrease it. Dermatophytes tend to produce an alkaline pH when growing on Sabouraud's medium, this is due to the deamination of amino acids and consequent formation of ammonia.[22] On the other hand, certain non-pathogenic molds such as *Penicillium*

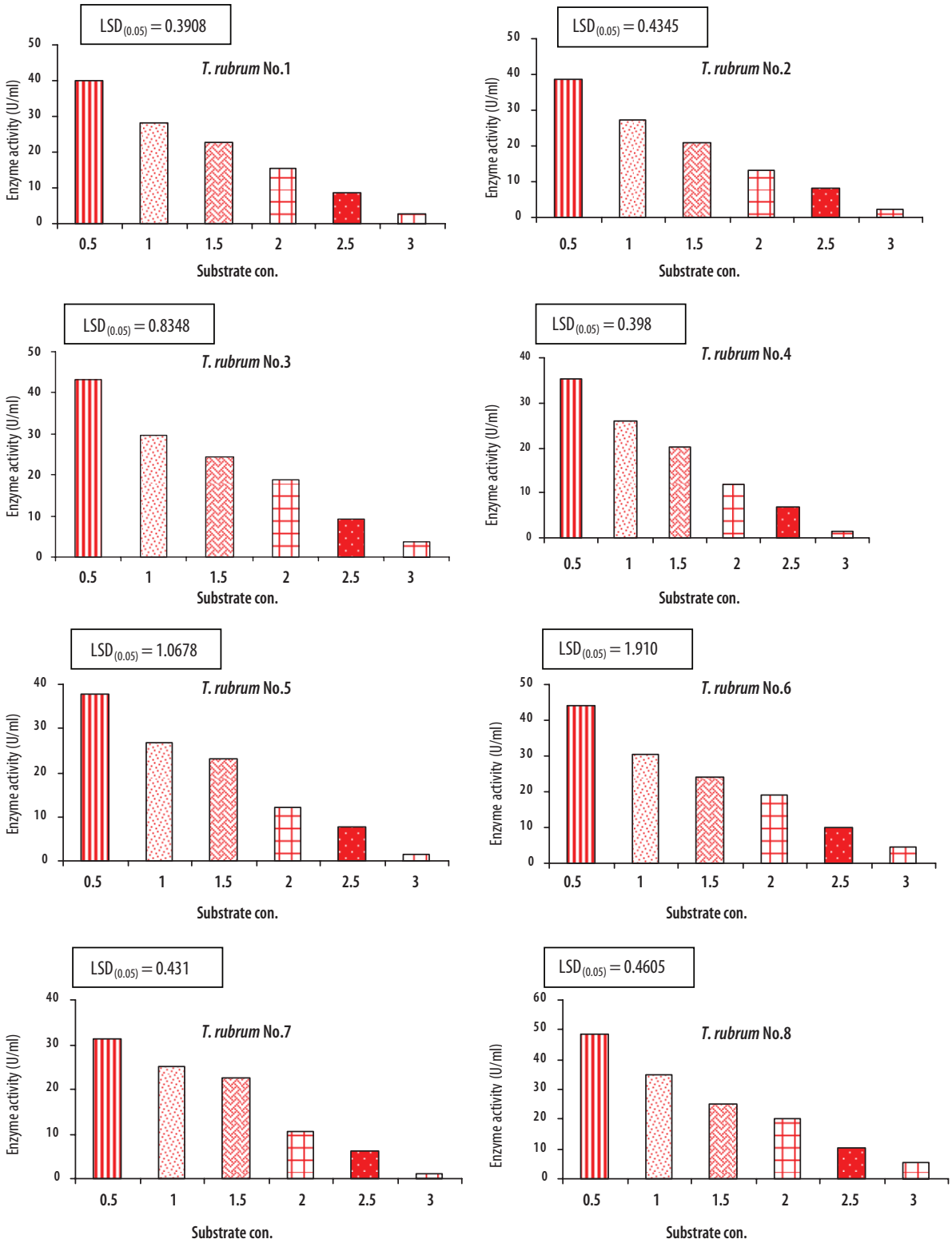

Fig. 4 **Effect of different substrate con. on the protease activity produced by *T. rubrum* isolates.**

and *Aspergillus* species, are known to shift the pH of the medium towards acidity.[23]

The pH of the culture media is important for mineral availability, enzyme activity, membrane function, growth and sporulation of keratinophilic fungi. Although the alteration that was found in medium was used for fungal growth, the cytoplasm remains conservative on the rate of hydrogen and hydroxyl ions because the plasma membrane that did not permit these ions to cross cytoplasm. pH is effective on enzymes found in cytoplasm that leads to the alteration of bioprocess of microorganisms and thus seem to be effective on either ion uptake or a loss to the nutrient medium.[24]

Sharma et al.,[19] observed that pH7 and 33°C temperature were the most suitable for the growth of *T. rubrum*. Our results are consistent with the

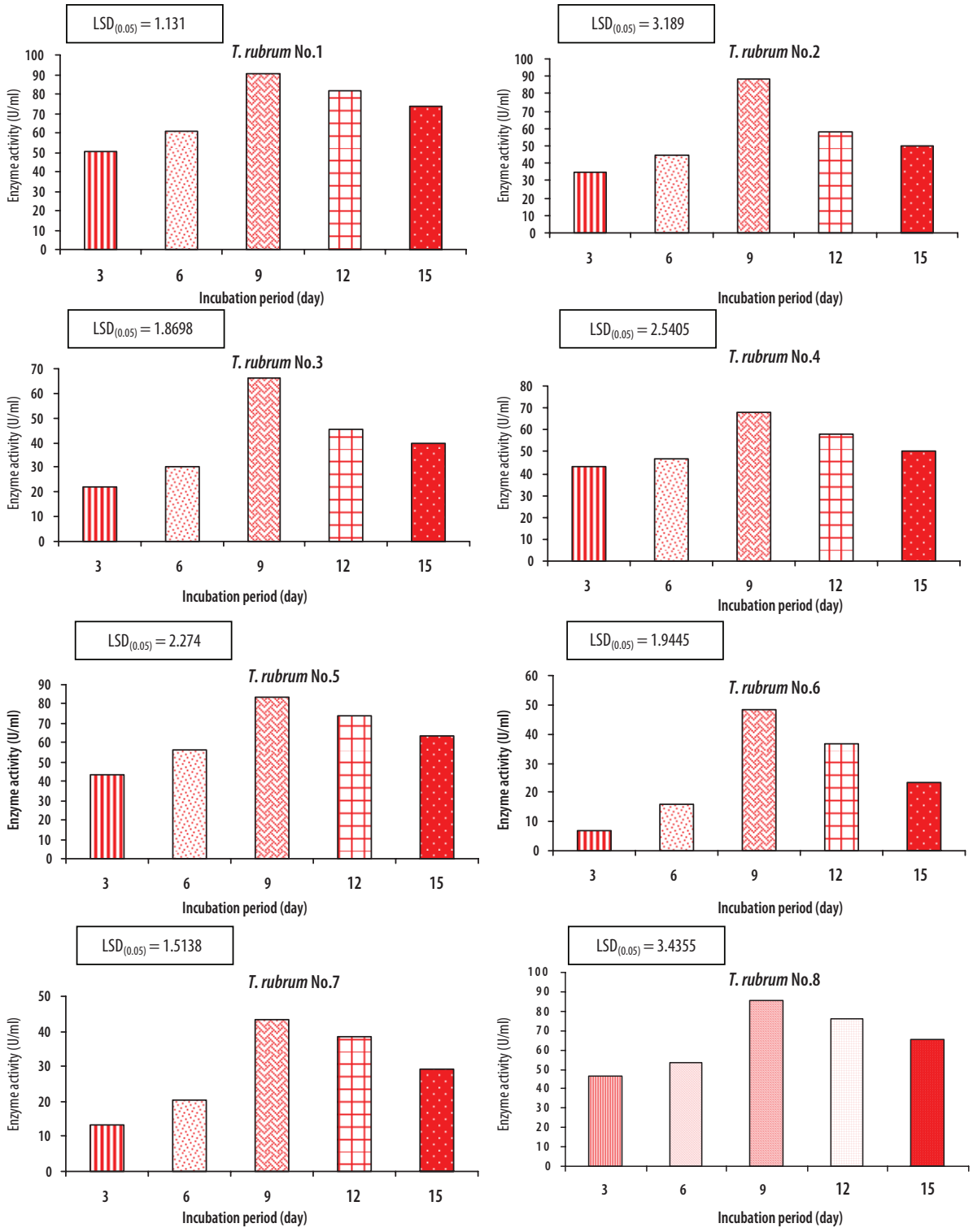

Fig. 5 **Effect of different incubation period (day) on the protease activity produced by *T. rubrum* isolates.**

results of Danew and Klossek,[25] who pointed the importance of pH in the growth of fungus.

Majority of fungi have been found to grow well at a pH range from 4.2–9.3. Usually too alkaline and too acidic solutions are not favorable for the growth of fungi. This might be because proteins have a tendency to develop lesser viscosity and simultaneously, their colloidal behavior changes in the sense that hydrolysis of proteins tends to form simpler products that results in the formation of colloidal particles of smaller dimension. This disturbance in the proper colloidal state of the cytoplasm hinders its normal function. Under such circumstances, the formation of the complex protein molecules becomes difficult resulting in the retardation of growth.[19]

The nature of a particular medium has a great role to play in the growth and sporulation of fungi. Kaul and Sambali[26] reported that keratinophilic fungi grow well in media rich in nitrogen and carbon contents. Maximum growth of

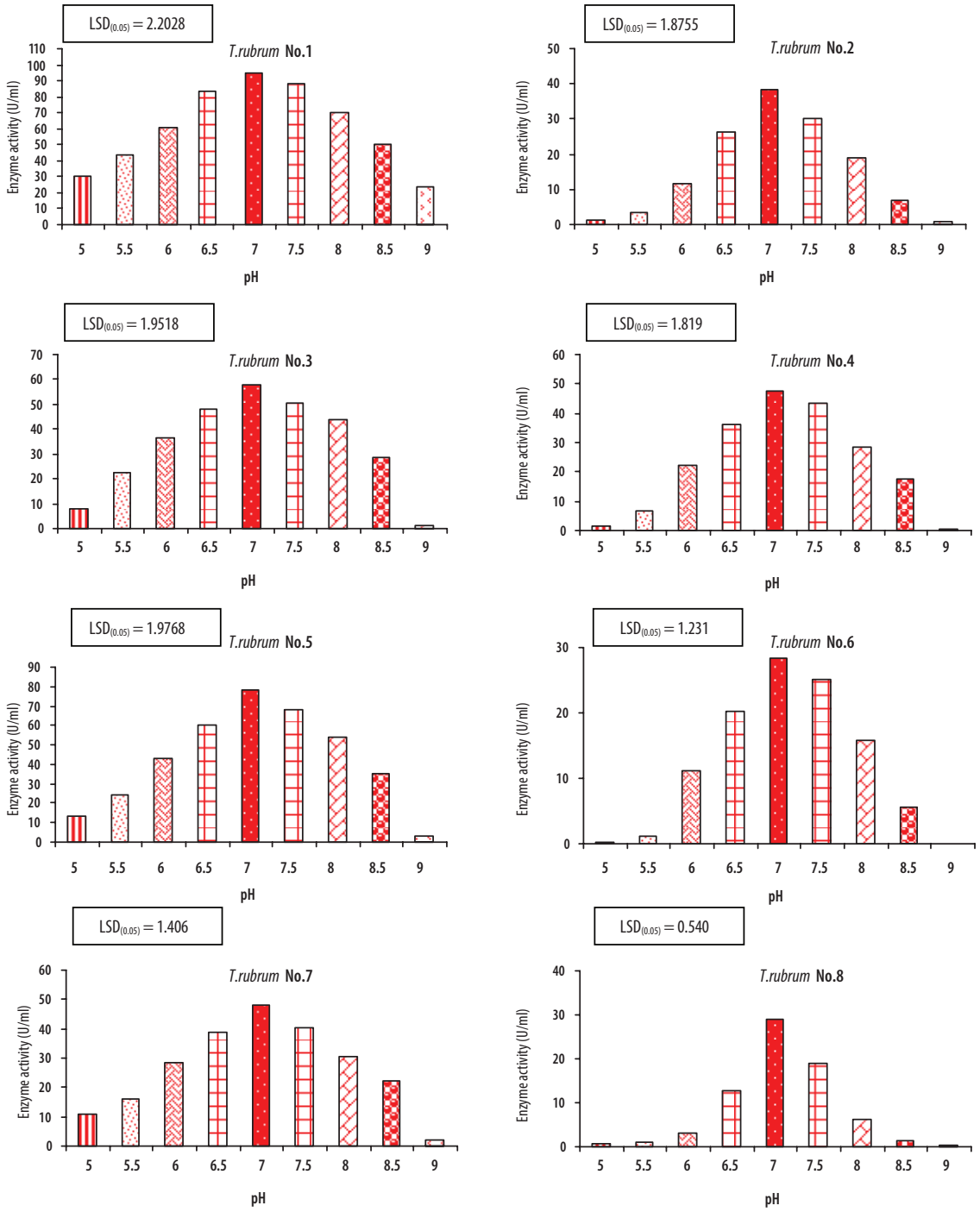

Fig. 6 **Effect of different pH on the protease activity produced by *T. rubrum* isolates.**

T. rubrum isolates were observed on SDA followed by PDA.

The reason for an increased rate growth of *T. rubrum* in the SDA medium was due to the presence of nutritional requirements for the growth of fungus in this medium such as the rate of glucose in the medium was 40 gm and peptone was 10 gm. Peptone contains a proportion of nitrogen which is reached to 13%.[27] It was believed that glucose was the most important determinant of suitable growth of fungi.[28] Our results agreed with Singh,[29] Jain[30] and Sharma & Sharma,[31] which found that SDA medium showed maximum growth and sporulation for all test fungi. Sharma[24] also agrees with the present investigation and suggested that the SDA medium is as an excellent source for almost all dermatophytic and keratinophilic fungi. The present study will help to maintain the fungus in the laboratory conditions for preparation of inocula for different studies concerning control of the human pathogen causing dermatophytic infections.

Effect of Some Culture Conditions on the Production of Protease

1. Incubation period

After protease production, the medium was incubated for different incubation periods (3, 6, 9, 12, 15 days), the production of protease reached maximum activity of *T. rubrum* isolates after 9 days

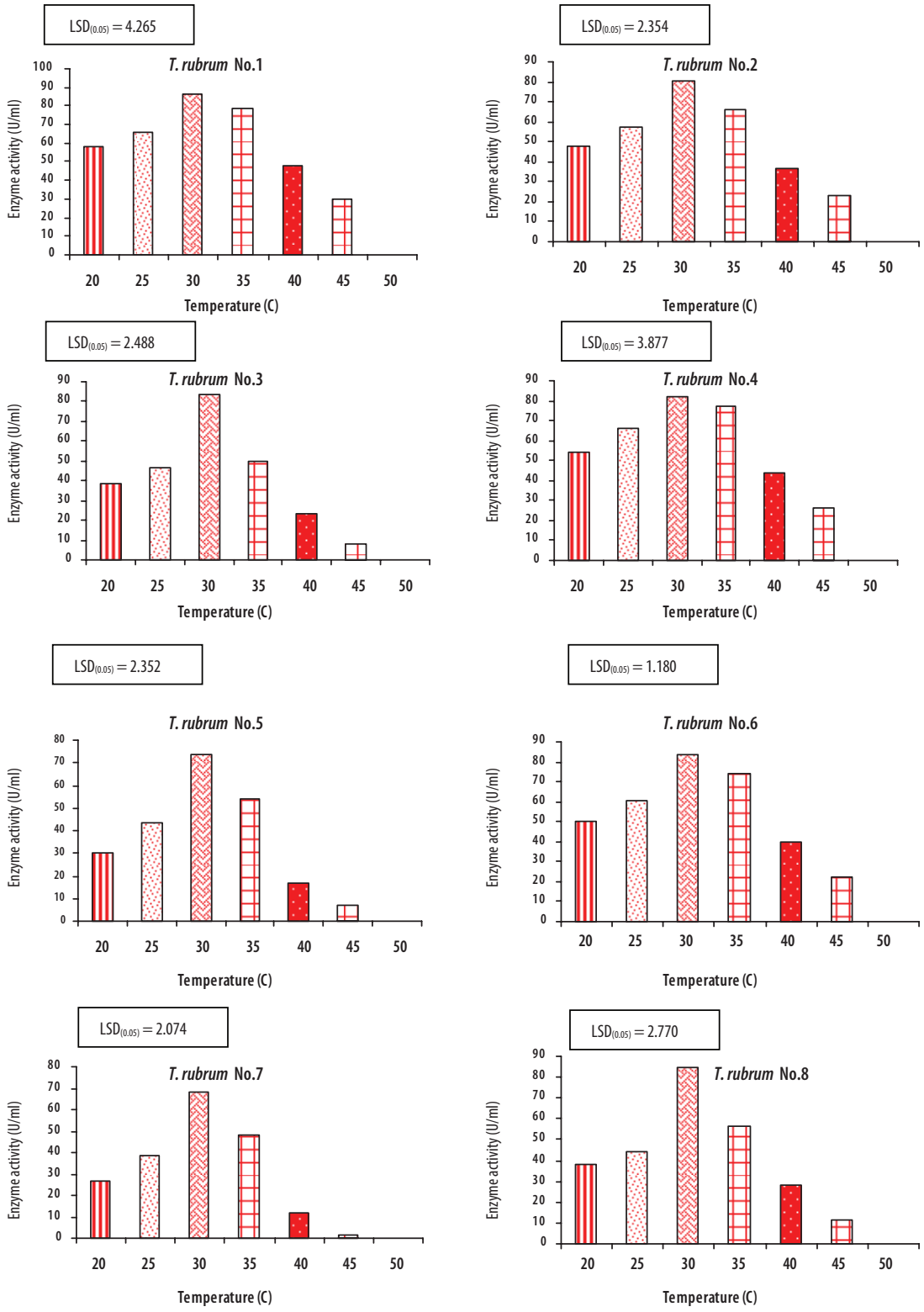

Fig. 7 **Effect of different temperature on the protease activity produced by *T. rubrum* isolates.**

of incubation. The optimal activity for protease of 8 isolates were 90.5, 88.5, 66.2, 67.8, 83.6, 48.5, 43.3 and 85.8 U/ml then a progressive decrease in protease production was observed when incubated for 12 and 15 days. The incubation period of 9 days was maintained to study the effect of other factors. These results was in agreement with Mahmood et al.[32] that showed 9 days was the best incubation period for the production of enzyme. Abdel-Hafez et al.[33] and El-Said[34] found that the maximum protease production by *T. rubrum* was observed after 8 days of incubation at 30°C. A progressive decrease in proteolytic activity occurred thereafter. Studies on other species of fungi have shown that in most cases proteolytic activity increases with the beginning of autolysis.[35,36] Apodaca and McKerrow[37] showed that during log phase growth most of the proteolytic enzyme of *T. rubrum* are repressible *in vitro* by small molecules and are likely

repressed during early growth *in vivo*. The entire complement of proteinases in *T. rubrum* tends to be produced constitutively during the stationary phase of growth *in vitro*.

Reduction in the production of enzyme was due to the cell which may reach to the decline phase, accumulation of waste materials and unavailability of nutrients.[38] Incubation time being an important factor has been responsible for optimum enzyme formation and it varies from one organism to another due to the variation in the lag and log phase of growth.[39]

2. Temperature

Temperature is a very important factor in the enzyme production since it plays a role in the structure of the enzyme and in the growth of microorganisms.[40,41] The optimum temperature for protease production by *T. rubrum* isolates in the present study was at 30°C which reached to 86.2, 80.1, 83.6, 82.1, 73.6, 83.4, 68.1 and 84.6 U/ml for eight tested isolates then reduced with a rise in the temperature. The results in this study agreed with the results reported by El-Said,[34] who reported that the optimum temperature for protease production from *T. rubrum* isolates was 30°C.

Decrease or increase in incubation temperature is an essential factor because of its importance in microorganism growth, metabolite production and suppression of cell viability.[42] Another possible reason could be due to the breaking down of enzyme at higher temperature as enzyme denature.[43]

The results of the study agreed with other studies which reported that most enzymes denaturated and lost its activity when temperature exceeds 35°C, also high temperature have affected the fungus growth and protease production. Also the studies indicated that the protease production of fungus decreases when incubation temperature was less or high than 30°C. Recent investigations have shown that proteases secreted by dermatophytes are similar to those of other fungi such as *Aspergillus* spp.[44–46]

3. pH

Acidity of the culture media is one of the most critical parameter that affected the production of proteases. pH is affected in the ionic state of amino acids that is responsible for the primary and secondary structure of enzyme and leads to affect the enzyme activity.[47]

The results in this study demonstrated that the optimum pH for proteases production was neutral range. Similarly for this work, Abdel-Hafez et al.[33] and El-Said[34] found that the maximum protease production by *T. rubrum* was within the range of pH 6–8. Asahi et al.[48] showed that the extra cellular proteinases of *T. rubrum* also have optimal proteolytic activity at neutral pH. But in other studies with *T. rubrum*, proteinase with a pH optimum of 4.5 was detected.[49] *T. rubrum* has been extensively studied by Apodaca and McKerrow[37] that showed to produce two strongly keratinolytic proteinases, as well as a poorly keratinolytic, trypsin- or chemotrypsin-like general proteinase. All these proteinases have a pH optimum of approximately 8. In a study of protease production during autolysis in different species of filamentous fungi, it was observed that autolysis occurred at pH values between 6.5–8.[50]

Human skin has a weak acidic pH and it is noteworthy that proteinases with an optimal activity under acidic conditions are reported to be important virulence factors in *T. mentagrophytes*.[51] Dermatophyte proteolysis results in the liberation of excess ammonium ion, raising the pH of the growth medium.[52] This reaction, an attribute relatively uncommon in fungi isolated clinically, has been used as the basis of screening media such as dermatophyte test medium.

4. Substrate concentration

The concentration of casein is an important factor for protease production. Enzyme production was found to influence with casein concentration. The results in this study agreed with Reddy and Saritha[53] who found that the suitable substrate concentration was 0.6 gram per 100 ml of production medium and Rajendran et al.[54] reported an initial substrate concentration of 0.5% of pectinase, this may be due to that in high substrate concentration no more enzymes are available free for carrying the reaction since this enzyme is constant.[55]

Further increase in substrate concentration resulted in drastic reduction in enzyme production, probably due to catabolic repression of biosynthesis[56] or a result of reduced mass transfer of oxygen by higher amount of solid substrate.[57]

The increase of substrate concentration leads to decrease in enzyme activity, this is probably due to the disintegration of some substrate compounds that play role as inhibitors for enzymes and decreased enzyme activity. While the small amounts of substrate concentration does not have any activity because of increase enzyme production, their results was in agreement with Teixeira et al.,[58] who showed the best pectin concentration for pectinase production from *Aspergilus japonica* was 0.5%. ■

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