Pak. J. Bot., 47(3): 1121-1127, 2015.

PRODUCTION OF RENNIN-LIKE ACID PROTEASE BY *MUCOR PUSILLUS* THROUGH SUBMERGED FERMENTATION

SUNILA DAUDI, HAMID MUKHTAR*, ASAD-UR-REHMAN AND IKRAM-UL-HAQ

Institute of Industrial Biotechnology, Government College University, Lahore-54000, Pakistan
**Corresponding Author's email: hamidmukhtar@gcu.edu.pk*

Abstract

The present study is concerned with the isolation and screening of *Mucor* species for the production of acid protease in shake flasks. Out of eight mould cultures evaluated, five were isolated from soil and three were provided from the Institute of Industrial Biotechnology, Government College University, Lahore. Of all the isolates tested, *Mucor pusillus* IHS_6 was found to be the best producer of rennin-like acid protease producing 75 U/ml of the enzyme. Different agricultural byproducts were evaluated as fermentation substrates and maximum enzyme synthesis (61 U/ml) was obtained when rapeseed meal was used as a substrate. Optimum pH and fermentation period for the production of protease were 5.5 (56U/ml) and 72 hrs (55U/ml), respectively. The production of protease by *Mucor pusillus* IHS_6 was also studied by adding different carbon and nitrogen sources to the fermentation medium. Fructose at a concentration of 1.5% (66 U/ml) and yeast extract at a concentration of 2% (68.2 U/ml) and ammonium chloride at a concentration of 0.1% (67U/ml) were found to be the best carbon and nitrogen (organic and inorganic) sources respectively. Spore inoculum at a concentration of 1% (68.4 U/ml) was found to be the best for protease production by *Mucor pusillus.* The fermentation broth was found to have strong milk clotting activity with 200 RU.

Key words: Milk clotting, Cheese, Protease, Fungi, Rennet.

Introduction

Proteases are found in a wide diversity of sources such as plants, animals and microorganisms but they are mainly produced by bacteria and fungi. The microbial proteases account for 60% of the worldwide enzyme commercialization (Gupta *et al.,* 2002). Fungi are an attractive source of proteases owing to the limited space required for their cultivation and their ready susceptibility to genetic manipulation. So, it is a group of great practical and scientific interest to biotechnologists. Many moulds synthesize and excrete large quantities of proteases into the surrounding media. A number of fungal genera such as *Aspergillus, Rhizopus, Mucor*, *Penicillium, Fusarium*, *Cephalosporium, Conidiobolus* and *Neurospora* are known to produce acid, alkaline or neutral proteases (Karuna & Ayyanna, 1993; Yongquan *et al.,* 1998; Baoying & Jianmin, 1998). These proteases are active over a wide pH (pH 4-11) and temperature range (30-70ºC).

Acid proteases of commercial importance are prepared exclusively from fungal sources and are tentatively divided into two subgroups by their physiological characteristics i.e; pepsin-like acid proteinases and rennin-like acid proteinases. Pepsin-like acid proteinases have usually been reported in the group of black aspergilli such as *Aspergillus niger, A. awamori*, *A. usamii* and *A. saitoi* but also occur in species of *Penicillium* and *Rhizopus* (Smail *et al.,* 1995; Yang & Lin, 1998; Haq *et al.,* 2003). Rennin-like acid proteinases are produced by strains of *Mucor miehei, Mucor pusillus* and *Trametes sanguinea* (Sathya *et al*., 2009). Studies on milk clotting enzymes have been carried out by many workers because of their industrial importance for use as rennin substitutes in cheese making. For this purpose various fungal strains including species from *Aspergillus*, *Penicillium* and *Mucor* have been exploited (Lima *et al*., 2008).

Limited availability of rennin has prompted many investigators in the last decades to search for rennin-like enzyme from microorganisms in general and fungi in particular. Of these fungi, only three strains belonging to *Mucor miehei, M. pusillus* and *Endothia parasitica* are used worldwide for the production of microbial rennet (Jiao *et al.,* 1992; Sekar *et al.,* 1998; Beyenal *et al*., 1999).

The main milk-clotting enzyme found in animal tissues, higher plants and microorganisms is rennin. The production of milk-clotting enzymes from microbial sources for use as rennin substitutes has been receiving increasing attention. For long time, calf rennet has been the traditional source of milk-clotting enzymes for cheese manufacturing but decreasing worldwide rennet supplies accompanied by ever increasing cheese production and consumption made necessary the utilization of rennet substitutes (Areces *et al*., 1992). It is used extensively in the dairy industry to produce a stable curd with good flavor. The specialized nature of the enzyme is due to its specificity in cleaving a single peptide bond in k-casein to generate insoluble para-k-casesin and C-terminal glycopeptide.

Peptide-hydrolysing enzymes (peptidases) play an important role in the food industry so the peptidase producing organisms are used as starters for the production of cheese. The peptidases produced by these microorganisms play a key role in the process of hydrolysis of milk casein into smaller peptides and free amino acids, which may contribute to the formation of special flavors of cheeses. The enzyme rennet can be used to catalyze the conversion of casein in milk to para-casein by removing a glycopeptide from the soluble casein. Para-casein further clots i.e., coagulates in the presence of calcium ions to form white, creamy lumps called the curd leaving behind the supernatant called the whey.

Present study is aimed at the production of rennin-like acid protease by a locally isolated fungal strain of *Mucor pusillus* and the indigenous production of the enzyme for its use in the local cheese producing industry.

Materials and Methods

Microorganisms and maintenance: Eight mould cultures belonging to genus *Mucor* including *M. pusillus* and *M. miehei* were taken from culture bank of Institute of Industrial Biotechnology, GC University, Lahore. All the strains were isolated from soil samples taken from different habitats and different localities. The strains were transferred and maintained on potato-dextrose-agar (PDA) slants. All the cultures were screened for the production of rennin-like acid protease using submerged fermentation.

Inocula preparation: Spores from 5 days old slants having profused growth and sporulation were wetted by adding 10ml of 0.005% solution of monoxol O.T (Di-octyl ester of sulphosuccinic acid). The spores were scratched with the help of sterilized wire loop for breaking the spore clumps and making a homogenous suspension. This spore suspension was used for inoculating the fermentation flasks.

Vegetative inoculum was prepared in 250 ml Erlenmeyer flask containing 50 ml of sterilized Vogel's medium consisting of (% w/v) trisodium citrate, 0.5; KH_2PO_4, 0.5; NH_4NO_3, 0.2; $(NH_4)_2SO_4$, 0.4; $MgSO_4$, 0.02; peptone, 0.1; yeast extract, 0.2 and 2 ml of separately autoclaved glucose solution (50%). Few glass beads also were added to the flasks to avoid the formation of mycelial pellets. After cooling, the flasks were inoculated with a loopful of spores from 72 hrs old slant. The flasks were placed in a rotary incubator shaker 200 rpm at 30ºC for 24 hrs. This mycelial suspension was then used to inoculate the fermentation medium.

Fermentation procedure: The fermentation experiments were carried out as batches in 250 ml Erlenmeyer flasks. The flasks containing 50 ml of the culture medium {(%, w/v) Soybean meal, 1.0; Glucose, 1.0; Polypeptone, 0.5; Yeast Extract, 0.2; KH_2PO_4, 0.1} were cotton plugged, autoclaved and inoculated with 1 ml of the inoculum as prepared earlier. After inoculation, the flasks were incubated at 30ºC for 72 hrs with shaking at a speed of 200 rpm in an orbital shaking incubator. After 72 hrs of incubation, contents of the flasks were filtered with Whattman filter paper # 44 and the filtrate was used for further analytical procedures.

The fermentation batches were run in triplicate and the mean of three values was reported in the results. During all the experiments, calibrated glassware and analytical grade chemicals were used.

Assay of protease activity: The assay of protease activity was carried out by the method of McDonald and Chen (1965) in which casein was used as a substrate. 1 ml of enzyme was incubated with 4 ml of 1% casein solution at 35ºC for 1 hr. The reaction was stopped and residual protein was precipitated by adding 5 ml of TCA solution. The tubes were then centrifuged at 6000 rpm for 5 min. After centrifugation, 1 ml of supernatant was mixed with 5 ml of alkaline reagent. Then 1 ml of 1N sodium hydroxide was added to make contents of the tubes alkaline. After at least 10 min, 0.5 ml of Folin and Ciocalteau reagent was added to each tube and the blue color produced was measured for OD at 700 nm using a UV-VIS spectrophotometer.

One unit of protease activity corresponds to an increase of 0.1 in optical density at 700 nm under the defined conditions.

Assay of milk clotting activity: The assay of milk clotting activity was carried using the method of Tubesha & Al-delaimy (2003). To the milk substrate pre-incubated at 35ºC, enzyme was added and mixed by inversion. The mixture was kept at 35ºC and the end point was marked by the appearance of milk clots. The unit of milk clotting activity was defined as the amount of enzyme that just started the clotting of milk substrate by the enzyme solution at 35ºC in 1 min. The amount of enzyme producing clotting in one minute is equal to 200 RU (Rennin units).

Results and Discussion

Screening of mould cultures: The data of Table 1 shows the screening of mould cultures for the production of acid proteases in shake flasks. Of all the cultures tested, *Mucor pusillus* IHS_6 gave maximum production of protease i.e. 75 U/ml. All the other strains also showed protease production with *Mucor miehi* IHS_7 as the second best producer (55.50 U/ml). Thus the strain *Mucor pusillus* IHS_6 was selected for further studies for the production of protease by submerged fermentation.

The production of milk clotting enzymes from different fungal cultures including *Penicillium* and *Aspergillus* have been reported (Ahmad & Ali, 1987) but very little work has been conducted for the production and application of rennin-like enzymes from *Mucor* spp. (Shaker & Brown, 1985) and none of any reports have indicated that the use of rennet from *Mucor* species is not safe for use in the cheese manufacturing. Therefore, the indigenous production of rennets from a locally isolated *Mucor pusillus* is of great significance for the cheese producing industry.

Rate of fermentation: The production of enzyme was increased with increasing time of incubation and highest enzyme activity (75 U/ml) was obtained after 72 hours of incubation (Fig. 1). It was also observed that increase in the incubation period after 72 hrs resulted in the decrease of enzyme activity. After that, the enzyme production and growth of the microorganism starts to decrease which can be attributed to the decrease in supply of nutrients (Romero, *et al.,* 1998). Karuna & Ayyanna (1993) reported that highest yield of protease by *Rhizopus oligosporous* was obtained after 72 hrs of incubation. Similarly, Ikasari & Mitchell (1994) have reported the maximum production of protease by *Aspergillus* sp. after 72 hrs of incubation. Tubesha & Al-Delaimy (2003) also reported that the optimum fermentation period for the production of acid proteases by *Mucor pusillus* is 3 days i.e., 72 hrs. The results of the present study are in accordance with the work reported by these workers.

Table 1. Screening of *Mucor* strains for protease production in shake flasks*.

S. No.	Mould culture	Enzyme activity (U/ml)	Milk clotting activity (RU)
1.	*Mucor* sp. IHS_1	20.50 ± 0.85^{h}	Nil
2.	*Mucor* sp. IHS_2	25.75 ± 1.19^{g}	Nil
3.	*Mucor* sp. IHS_3	40.00 ± 0.75^{d}	140 ± 05^{b}
4.	*Mucor* sp. IHS_4	35.75 ± 1.27^{e}	80 ± 05^{d}
5.	*Mucor* sp. IHS_5	30.00 ± 1.08^{f}	50 ± 05^{f}
6.	*Mucor pusillus* IHS_6	75.00 ± 0.81^{a}	165 ± 5^{a}
7.	*Mucor miehi* IHS_7	55.50 ± 1.05^{b}	60 ± 08^{e}
	Mucor miehi IHS_8	45.50 ± 1.00^{c}	120 ± 06^{c}

Each value is a mean of three parallel replicates. ± indicates the standard deviation from the mean value.
Values differ significantly at $p \leq 0.05$.
*Incubation temperature = 30ºC, incubation period = 72 hrs, initial medium pH = 5.0

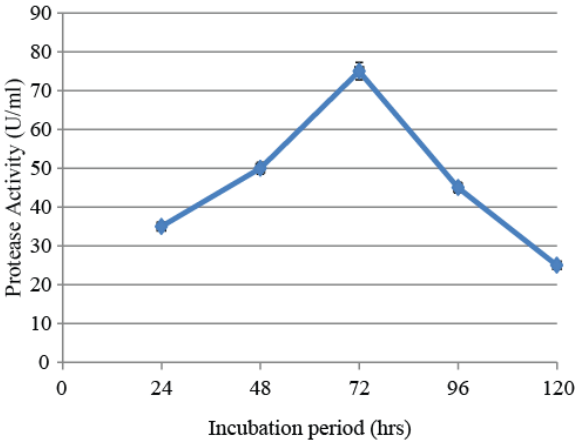

Fig. 1. Effect of rate of fermentation on protease production by *Mucor pusillus* IHS_6 in shake flasks*.
Each value is a mean of three parallel replicates. Error bars indicates the standard deviation from the mean value.
Values differ significantly at $p \leq 0.05$.
*Incubation temperature = 30ºC, initial medium pH = 5.0

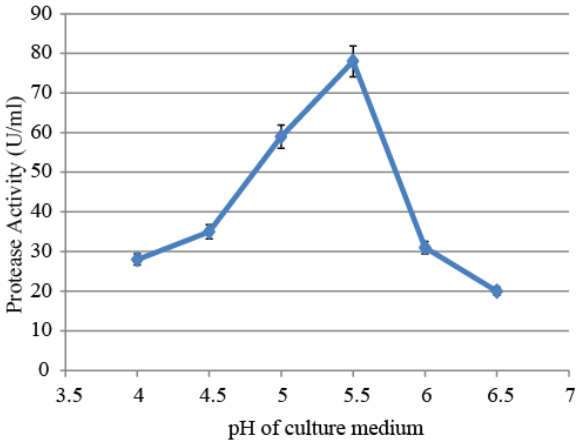

Fig. 2. Effect of pH of culture medium on protease production by *Mucor pusillus* IHS_6 in shake flasks*.
Each value is a mean of three parallel replicates. Error bars indicates the standard deviation from the mean value.
Values differ significantly at $p \leq 0.05$.
*Incubation temperature = 30ºC, incubation period = 72 hrs

Effect of initial pH of the medium: Fig. 2 shows the effect of initial pH of the fermentation medium on the production of acid protease by *M. pusillus* in shake flasks. The mould culture produced maximum amount of protease at pH 5.5 which was 78 U/ml. The pH of the culture media has marked effect on the type and amount of enzyme produced. One organism may secrete variable amounts and types of enzymes depending upon the pH and composition of medium. Change in pH may also cause denaturation of enzyme resulting in loss of catalytic activity. Like other proteins, enzymes possess many ionizable groups, so pH changes may also alter the conformation of the enzyme, the binding of the substrate and the catalytic activity of the groups at the active site of the enzyme. Change in ionic state of substrate may result in the formation of charged particles which may not correspond with ionic active sites of the enzyme. Yegin *et al.* (2010) have reported a medium pH of 4.5 as the best for protease production by *M. mucedo* in submerged fermentation.

Effect of incubation temperature: The effect of incubation temperature on the production of protease by *M. pusillus* was studied by incubating the flasks at different temperatures i.e. 20, 25, 30, 35, 40 and 45ºC for 72 hrs. The optimum temperature for enzyme production was found to be 30ºC where maximum production of protease (78 U/ml) was obtained (Fig. 3). As the temperature was increased above 30ºC, the enzyme production was decreased. It may be due to the fact that rate of all the physiological processes increases by increasing the temperature but beyond certain limits it starts decreasing. Because enzymes are sensitive to temperature, rise of temperature within certain limits has the effect of increasing the activity of enzymes but very high temperatures are destructive. An enzyme loses its catalytic properties at high temperature due to stretching and final breaking of weak hydrogen bonds present in enzyme structure, this results in change in the nature of the enzyme. Some other workers have also reported 30ºC to be the optimum temperature for production of rennet by *Mucor pusillus* in submerged culture fermentation (Khan *et al.*, 1979).

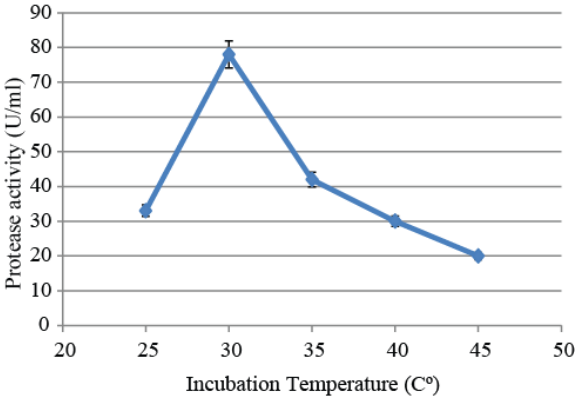

Fig. 3. Effect of incubation temperature on protease production by *Mucor pusillus* IHS_6 in shake flasks*.
Each value is a mean of three parallel replicates. Error bars indicates the standard deviation from the mean value. Values differ significantly at $p \leq 0.05$. *incubation period = 72 hrs, medium pH=5.5.

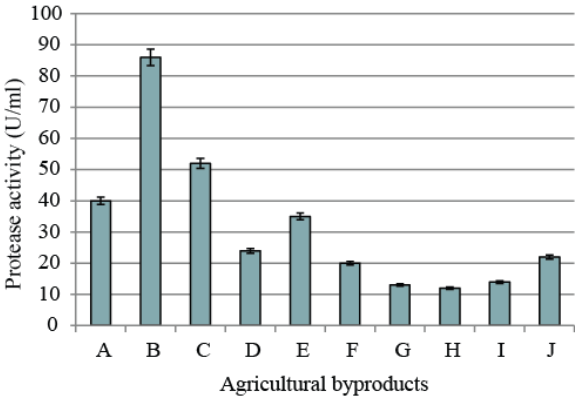

Fig. 4. Evaluation of different agricultural byproducts for protease production by *Mucor pusillus* IHS_6 in shake flasks*
Each value is a mean of three parallel replicates. Error bars indicates the standard deviation from the mean value. Values differ significantly at $p \leq 0.05$. *Incubation temperature = 30ºC, incubation period = 72 hrs, medium pH = 5.5. Symbols used: A = Gram husk; B = Rape seed meal; C = Corn Gluten meal; D = Cotton seed meal; E = Soybean meal; F = Guar meal; G = Wheat flour; H = Almond meal; I = Sunflower meal; J = Wheat bran

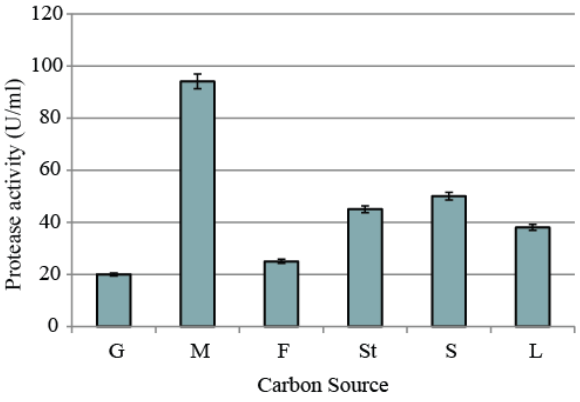

Fig. 5. Effect of different carbon sources on protease production by *Mucor pusillus* IHS_6 in shake flasks *.
Each value is a mean of three parallel replicates. Error bars indicates the standard deviation from the mean value. Values differ significantly at $p \leq 0.05$. *Incubation temperature = 30ºC, incubation period = 72 hrs, medium pH = 5.5. Symbols used: G = Glucose; M = Maltose; F = Fructose; St = Starch; S = Sucrose; L = Lactose

Screening of agricultural byproducts: Different agricultural byproducts such as gram husk, rape seed meal, corn gluten meal, cotton seed meal, soybean meal, guar meal, wheat flour, almond meal, sunflower meal and wheat bran as sources of protein, carbohydrate and minerals were evaluated for the production of rennin-like acid protease by *Mucor pusillus* IHS_6 in submerged fermentation (Fig. 4). Of all the substrates examined, rapeseed meal supported maximum growth of microorganism with maximum production of enzyme (86 U/ml). Gram husk and corn gluten meal also supported considerable production of acid protease (40 & 52 U/ml) by the microorganism but other agricultural byproducts did not resulted in good titers of the enzyme.

The reason of highest yield was due to the fact that rape seed meal provided an adequate source of protein, carbohydrate and minerals needed by the organism for the biosynthesis of proteases. Rape seed meal is a high protein and a good energy byproduct of rapeseed (*Brassica napus*) after oil extraction and is widely and freely available in Pakistan. Yeoman & Edwards (1997) have also used rape meal derived fermentation media for the production of protease by *Streptomyces thermovulgaris.*

Selection of carbon source: Different carbon sources were evaluated for the production of acid protease by *Mucor pusillus* in submerged fermentation (Fig. 5). Carbon sources such as starch, fructose, lactose, maltose, glucose and sucrose were added to the fermentation medium which gave enzyme activities of 20, 94, 25, 45, 50 and 38 U/ml, respectively. As shown in the results, highest yield of protease (94 U/ml) was observed when fructose was used as a carbon source in the fermentation medium.

Carbon source is one of the important factors affecting enzyme production and it has been noticed that the absence of a proper carbohydrate (c-source) in the medium results in a dramatic decrease in enzyme production (Gajju *et al.*, 1996), so a carbon source is always an essential component of a fermentation medium. Sutar *et al*. (1990) reported that fructose was best carbon source for protease production from *Conidiobolus coronatus*.

The concentrations of fructose in the fermentation medium was also optimized and it was found that maximum activity of protease (94 U/ml) from *Mucor pusillus* IHS_6 was obtained when fructose was added to the fermentation medium at a concentration of 1.5% (Fig. 6). The lesser concentrations did not support more production of enzyme due to deficiency of carbon source needed by the fungus while higher concentration of fructose might have some inhibitory action on the growth of the microorganism. The decrease of protease production at higher fructose concentration suggests that, at least in part, the synthesis and secretion of protease is regulated by carbon catabolite repression. Thus, fructose at a concentration of 1.5% was found as the best carbon source for the production of rennin-like acid protease by *M. pusillus* IHS_6.

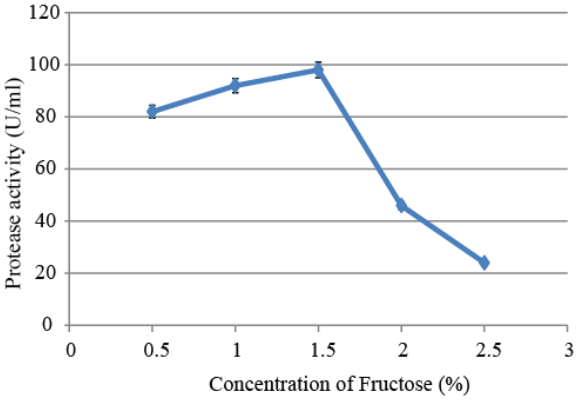

Fig. 6. Effect of concentration of fructose on protease production by *Mucor pusillus* IHS_6 in shake flasks*.
Each value is a mean of three parallel replicates. Error bars indicates the standard deviation from the mean value. Values differ significantly at p≤0.05. *Incubation temperature = 30ºC, incubation period = 72 hrs, medium pH = 5.5, carbon source = fructose

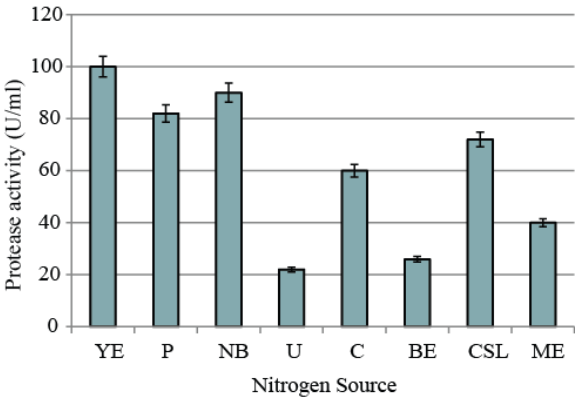

Fig. 7. Effect of different Nitrogen sources on protease production by *Mucor pusillus* IHS_6 in shake flasks*.
Each value is a mean of three parallel replicates. Error bars indicates the standard deviation from the mean value. Values differ significantly at p≤0.05. *Incubation temperature = 30ºC, incubation period = 72 hrs, medium pH = 5.5
Abbreviations:_ YE = Yeast Extract; CSL = Corn Steep Liquor; P = Polypeptone; NB = Nutrient broth; ME = Meat Extract; BE = Beef Extract; U = Urea; C = Casein

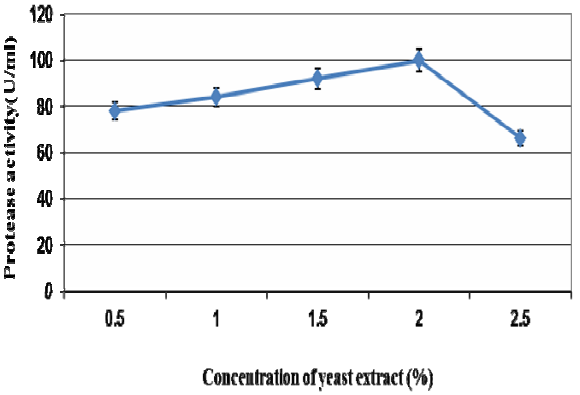

Fig. 8. Effect of concentration of yeast extract on protease production by *Mucor pusillus* IHS_6 in shake flasks*.
Each value is a mean of three parallel replicates. Error bars indicates the standard deviation from the mean value.
Values differ significantly at p≤0.05. *Incubation temperature = 30ºC, incubation period = 72 hrs, medium pH = 5.5

Selection of nitrogen sources: Different nitrogen sources, in the fermentation medium, were evaluated for the production of protease by *Mucor pusillus* IHS_6 (Fig. 7). Nitrogen sources such as yeast extract, polypeptone, nutrient broth, urea, corn steep liquor, meat extract, casein and beef extract were added to the fermentation flasks and yeast extract was found to be the best nitrogen source for enzyme production in shake flasks with enzyme production of 100 U/ml. Yeast extract was followed by nutrient broth as the second best nitrogen source for protease production (90 U/ml). All the other nitrogen sources gave lesser amount of enzyme.

The concentration of yeast extract in the fermentation medium (0.5-2.5%) was also optimized and it was found that maximum activity of protease (100 U/ml) from *Mucor pusillus* was obtained when yeast extract was added to the fermentation medium at a concentration of 2% (Fig. 8). The lesser concentration did not show more production of enzyme due to deficiency of nitrogen source needed by the fungus while higher concentration of nitrogen might have some inhibitory action on the growth of the microorganisms due to nitrogen repression.

Different inorganic nitrogen sources such as ammonium chloride, sodium nitrate, potassium nitrate, ammonium sulphate and ammonium nitrate were also evaluated for the production of acid proteases by *Mucor pusillus* IHS_6. All the inorganic nitrogen sources were added to the culture medium at a concentration of 0.1%. The data of figure 9 shows the results, which depicts that the maximum biosynthesis of enzyme by microorganism (104 U/ml) was obtained when culture medium was supplemented with ammonium chloride. The order of production of the enzyme with other inorganic nitrogen sources were 60 > 55 > 50 > 45. This showed that ammonium chloride was the nutritional requirement of the organism for enzyme production or secretion. It was also reported by Bidochka & Khachatourians (1987) that ammonium chloride acted as a best inorganic source for protease production by *Beauveria bassiana*.

Effect of inoculum type and size: The types of inocula tested for the production of protease were spore and vegetative inoculum. The results showed that the maximum amount of enzyme (104 U/ml) was produced when spore inoculum was used whereas vegetative inoculum resulted in the decreased production of protease in shake flasks (Fig. 10). The type of inoculum has marked effect on the production of proteases and an appropriate spore inoculum is essential for maximum enzyme production. Highest yield from spore inoculum shows that sufficient quantity of mycelium was formed from it, which produced optimum level of enzyme. In case of vegetative inoculum, the amount of mycelium was increased rapidly which consumed majority of the substrate from the fermentation medium in a rapid progression and also the toxins produced as a result decreased the enzyme synthesis. Mukhtar & Haq (2009) also reported that spore inoculated fermentation medium gave much higher yields from *Penicillium chrysogenum* than in fermentation medium seeded with vegetative inoculum.

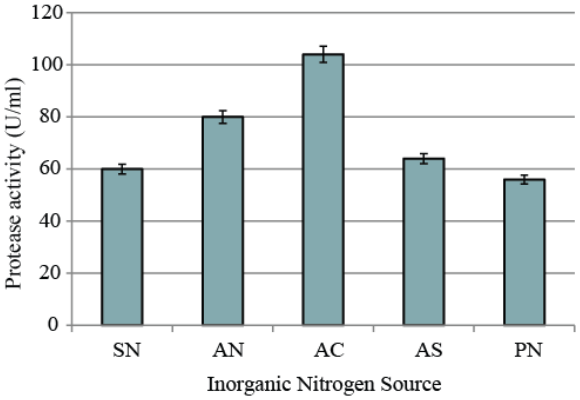

Fig. 9. Effect of inorganic nitrogen source on protease production by *Mucor pusillus* IHS_6 in shake flasks*.
Abbreviations: AC = Ammonium chloride; SN = Sodium nitrate; PN = Potassium nitrate; AS = Ammonium sulphate; AN = Ammonium nitrate
Each value is a mean of three parallel replicates. Error bars indicates the standard deviation from the mean value.
Values differ significantly at p≤0.05. *Incubation temperature = 30ºC, incubation period = 72 hrs, medium pH = 5.5

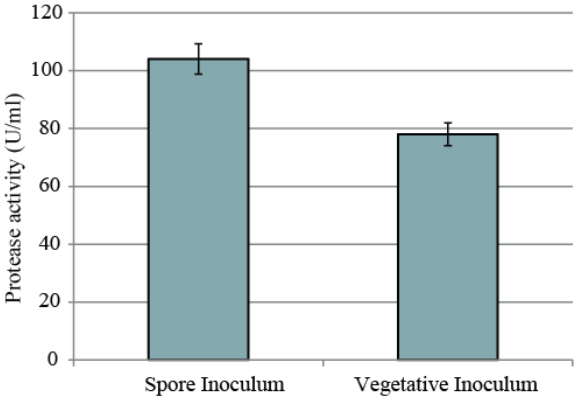

Fig. 10. Effect of type of inoculum on protease production by *Mucor pusillus* IHS_6 in shake flasks*.
Each value is a mean of three parallel replicates. Error bars indicates the standard deviation from the mean value. Values differ significantly at p≤0.05. *Incubation temperature = 30ºC, incubation period = 72 hrs, medium pH = 5.5

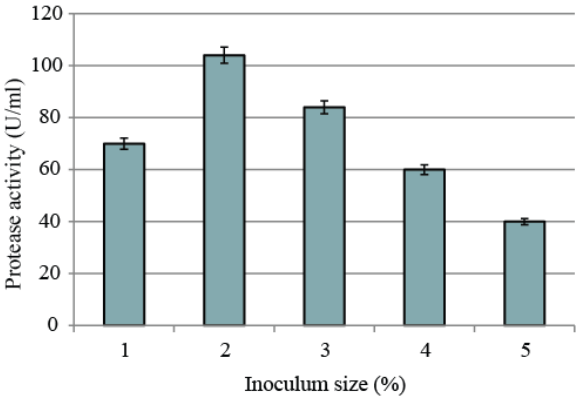

Fig. 11. Effect of size of inoculum on protease production by *Mucor pusillus* IHS_6 in shake flasks*.
Each value is a mean of three parallel replicates. Error bars indicates the standard deviation from the mean value. Values differ significantly at p≤0.05. *Incubation temperature = 30□C, incubation period = 72 hrs, medium pH = 5.5

The size of inoculum ranging from 1-5 % (v/v) was also evaluated for the production of protease by *Mucor pusillus* (Fig 11). The results showed that maximum amount of enzyme (102 U/ml) was produced when 1.0 ml (2%) of inoculum was used to inoculate the fermentation flasks. Further increase in volume of the inoculum resulted in the decreased enzyme production. An appropriate inoculum size is essential for maximum production of enzyme through submerged fermentation. Highest yield at 1.0 ml of inoculum lies in the fact that a sufficient quantity of mycelium was formed from this amount of mycelium, which produced maximum level of enzyme in the broth. Size of inoculum also determines the formation of filamentous or pellet growth of the organisms in shake flasks which in turn may affect the enzyme production and secretion by the fungus. *Mucor pusillus* IHS_6 yielded pellet growth with a small size of inoculum such as less than 1%. The decrease in enzyme production by increasing the size on inoculums was due to the fact that the mycelia started to consume rapidly most of the substrate in a rapid fashion for its growth purposes which resulted in decreased enzyme yield. Moreover, toxins are also produced in the fermentation broth by the rapid death of older cells. Similar results have also been reported by Haq *et al*. (2003) who studied the biosynthesis of protease by *Penicillium chrysogenum* using 2% (v/v) of spore inoculum.

Milk clotting activity and cheese production: The enzyme extract was centrifuged at 6000 rpm for 10-15 min. After centrifugation it was membrane filtered using cellulose acetate filters of 0.45µm so that no spores or mycelial fragments are left in the enzyme extract. The extract was assayed for milk clotting activity using a milk substrate. It was found that the enzyme produced by *Mucor pusillus* IHS_6 had strong milk clotting activity showing 200 RU (Table 2). The ratio of milk clotting activity to protease activity was 2.667 and having a protein content 64.85µg/ml.

For the application of the enzyme for cheese production, 1 liter of milk was pre-incubated at 35ºC for 5 min before the addition of enzyme. After addition of enzyme, the end point was marked by the appearance of milk clots i.e. curd formation. It was left for 24 hrs for further coagulation. After 24 hrs it turned into a more solid curd leaving behind the supernatant called the whey. Curd was separated from the whey, compressed and left for more seven days at 20ºC. After that it was observed that it turned to a solid cheese. The yield of cheese was also calculated, white curd cheese produced by the crude enzyme extract from *Mucor pusillus* IHS_6 was found to be 120 g from 1 liter cow's milk.

Table 2. Milk clotting activity of the proteolytic enzyme produced by *Mucor pusillus* IHS_6 and its application for cheese production.

Proteolytic activity (U/ml)	Milk clotting activity (RU)	MCA / PA	Protein content (µg/ml)	Cheese yield (g/l)
75	200	2.667	64.85	120

References

Ahmad, F.A. and S. Ali. 1987. Preparation and some properties of a partially purified rennin-like enzyme from *Penicillium expansum, Biological Wastes*, 20(1):35-41.

Areces, L.B., M.J. Biscoglio de Jiménez, M.A.A. Parry, E.R. Fraile, H.M. Fernández and O. Cascone. 1992. Purification and characterization of a milk-clotting protease from *Mucor baciliformis. App. Biochem. Biotechnol.,* 37: 283-294.

Baoying, T. and C. Jianmin, 1998. Selection of a acid resistant protainase – producing strain and its fermentation conditions. *Shipin. Yu Fajiao Gongye,* 24 (3): 16-19.

Beyenal H., S. Sekar, B. Salih and A. Tanyolac. 1999. The effect of D-glucose on milk-clotting activity of Mucor miehei in a chemostat with biomass retention. *J. Chem Technol Biotech*., 74: 527-532.

Bidochka, M.J. and G.G. Khachatourians. 1987. Purification and properties of an extracellular protease produced by the entomopathogenic fungus *Beauveria bassiana*. *Appl. Environ. Microbiol.,* 53(7):1679-1684.

Gajju, H., T.C. Bhalla and H.O. Agarwal. 1996. Thermostable alkaline protease from thermophilic *Bacillus coagulans PB-77. Ind. J. Microbiol*., 36: 153-155.

Gupta, R., Q.K. Beg and P. Lorenz. 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl. Microbiol Biotechnol*., 59: 15-32.

Haq, I., H. Mukhtar, S. Daudi, S. Ali and M.A. Qadeer. 2003. Production of proteases by a locally isolated mould culture under lab conditions. *Biotechnol*., 2(1): 30-36.

Ikasari, L. and D.A. Mitchell. 1994. Protease production by *Rhizopus oligosporous* in solid-state fermentation. *J. Microbiol. Biotechnol*., 10: 320-324.

Jiao Q., S. Shijun and G. Mang. 1992. SIN. Biosynthesis and properties of milk-clotting from *Mucor pusillus. Acta. Microbial.,* 32 30-35.

Karuna, J. and C. Ayyanna. 1993. Production of semi-alkaline protease enzyme from *Aspergillus* spp. Proceedings of the Ninth National Convention of Chemical Engineers and International Symposium on Importance of Biotechnology in coming decades. Viskhapatnam India, pp. 8-11.

Khan M.R., J.A. Blain and J.D.E. Patterson. 1979. Extracellular protease of *Mucor pusillus*. *Appl. Environ. Microbiol.,* 37(4):719.

Lima, C.J.B., M. Cortezi, R.B. Lovaglio, E.J. Ribiero, J. Contiero and E.H. Aurajo. 2008. Production of Rennet in Submerged Fermentation with the Filamentous Fungus *Mucor miehei* NRRL 3420. *World Applied Sci. J.,* 4(4): 578-585.

McDonald, C.E. and L.L. Chen. 1965. Lowry modification folin reagent for determination of proteinase activity. *Annals Biochem.,* 10: 175.

Mukhtar, H. and I. Haq. 2009. Production of acid protease by *Aspergillus niger* using solid state fermentation. *Pak. J. Zool*., 41(4):253-260.

Romero, F., L.A. Garcia and M. Diaz. 1998. Protease production from whey at high concentration by *Serratia marcescens. Resour. Environ. Biotechnol*., 2(2): 93-115.

Sathya, R., B.V. Pradeep, J. Angayarkanni, and M. Palaniswami. 2009. Production of milk clotting protease by a local isolate of *Mucor circinelloides* under SSF using Agro-industrial wastes, *Biotechnol. Bioprocess Eng*., 14: 788-794.

Sekar S., H. Beyenal, F. Ayhan and A. Tanyolac. 1998. Production of microbial rennin from Mucor miehei in a continuously fed fermenter. *Eng. Microb. Technol*., 23: 469-474.

Shaker, K.A. and R.J. Brown. 1985. Proteolytic and milk clotting fractions in milk clotting preparations, *J. Dairy Sci.,* 68(8): 1939-42.

Smail, T., O. Salhi and J.S. Knap. 1995. Solid state fermentation of carob pods by *Aspergillus niger* for protein production: effect of partical size. *J. Mircobial. Biotechnol.,* 11: 171-173.

Sutar, M., M.C. Srinivasan and H.G. Vartak. 1990. Production of an alkaline proteinase from *Conidiobolus coronatus. World J Microbiol. Biotechnol.,* 8: 254-258.

Tubesha, Z.A. and K.S. Al-Delaimy. 2003. Rennin-like milk coagulant enzyme produced by a local isolate of *Mucor, International J. Dairy Technol.,* 56(4): 237-41.

Yang, F.C. and I.H. Lin. 1998. Production of acid protease using thin stillage from a rice-spirit distillery by *Aspergillus niger*. *Enzyme Microbial Techonol*., 23(6): 397-402.

Yegin, S., M. Fernández-Lahore, U. Guvenc and Y. Goksungur. 2010. Production of extracellular aspartic protease in submerged fermentation with *Mucor mucedo* DSM 809. *Afr. J. Biotechnol*., 9(38): 6380-86.

Yeoman, K.H. and C. Edwards. 1997. Purification and characterization of the protease enzymes of *Streptomyces thermovulgaris* grown in rapemeal-derived media, *J. Applied Microbiol.,* 82: 149-156.

Yongquan, L. and H. Wengian. 1998. Study on sheep pert bating with acid protease, *Zhonggvo Pige,* 27: 16-17.

(Received for publication 10 March 2014)