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Study the influence of nitrogen on rennin production by fungi *Rhizomucor miehei* using solid-state fermentation

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Abstract. The effect of different nitrogen resources on the biosynthesis of milk clotting enzyme by *Rhizmucor miehei* was studied under solid state fermentation using wheat bran as base medium. Urea, peptone, albumin, casein, yeast extract were added with different concentrations (1%-10%). The response parameters were the ratio of milk clotting activity (MC) to proteolytic activity (PA) and protein content. The highest enzyme yield was achieved with casein at a rate of 2% w/w followed by 2% yeast extract, 1% albumin, 1% peptone, and 1% urea with values 5.6, 4.9, 4.2, 4, 3 mg/mL, respectively. Maximum enzyme activity (MCA/PA) was 50.4, 44.1, 37.8, 36, 27 SU for casein, yeast extract, albumin, peptone, and urea, respectively.

Keywords: Rennin; Protease; *Rhizomucor miehei*; Solid state fermentation; Nitrogen.

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Introduction

Rennet enzyme (rennin) is used in the manufacture of cheese, which is an acid protease, the effect of acid protease appears in the process of milk coagulation through two stages: in the first stage or enzymatic, casein hydrolyzed by rennin to produce para-casein which form the curd in the second phase (non-enzymatic). Milk clotting enzyme derived from the fourth stomach of and weaned calves and the crude extract called rennet, its purified active components called rennin.

The increase in global cheese production in recent years in association with sharp decrease in calf rennet lead to search for alternative rennet substitute, many enzymes from plant and animal sources used as substitute and which showed in majority unsatisfactory properties, in addition to microbial substitute which has wide acceptable. Many of Microorganism give the enzyme rennin, but it is not suitable as substitutes for rennet since it should satisfy the following requirements; its proteolytic activity should be similar to calf rennet, should be able to curdle milk at the same conditions (calcium ion concentration, pH and temperature), effective coagulation of milk without undue hydrolysis of curd, proper flavor. It should not cause unwanted bacterial growth, absence of toxins and pathogens, should not cause unwanted antibiotic activity.

Studies have been conducted on some classes of bacteria and fungi, *Aspergillus oryzae, Irpes lactis* (Neelakantan et al., 1999; Ire et al., 2011; Nouani et al., 2011), *Rhizopus* sp (Sumantha et al., 2006; Gais et al., 2009).

The bacterial proteases is not suitable because of its high nonspecific proteolysis, studies recently focused on three fungal strains to produce microbial rennet *Rhizomucor miehei*, *Endothia parastica*, *Rhizomucor pussilus* (Thakur et al., 1990; Kazemi-Vaysari et al., 2002; Silveira et al., 2005; Farshad et al., 2013).

Currently most of the microbial rennet products in the world is produced using these three strains and sold under the trade names such as hannilase, rennilase, formase, miki, maryzme, modilase (*Rhizomucor miehie*), noury, meito, emporase (*Rhizomucor pussilus*), suparen, sure curd (*Endoparthia parastica*) (Neelakantan et al., 1999; Tiwari, 2003).

The protease of molds *Rhizomucor miehei*, *Rhizomucor pussilus* split the same peptide bonds, Phe 105-Met 106 which chymosin split in kappa casein, protease of *Endothia parastica* split the bond Ser104-Phe105 (Tiwari, 2003).

Zygomicetes *Rhizomucor miehei* and *Rhizomucor pussilus* produce a coagulant with characteristics that are very similar to commercial rennet, producing high coagulation and production rates (Lima et al., 2008).

Rhizomucor pussilus extract is more proteolytic than calf rennet or *Rhizomucor miehei* extract, the clotting ability of its extract is three times of that in calf rennet, and not specific as *Rhizomucor miehei* extract, and tends to give hard curd because of its higher proteolytic activity and the curd tends to lose fat into whey, which make cheese yield lower, and produce bitter flavor in ripe cheese (Tiwari, 2003).

The protease of *Rhizomucor miehei* is the preferred substitute for calf rennet because of its specificity in splitting similar peptide bonds, high ratio of milk coagulation activity, identical calcium requirements and good cheese quality (Kazemi-Vaysari et al., 2002) and low incidence of bitter flavor in cheese curd (Tiwari, 2003).

The protease of *Rhizomucor miehei* is acid aspertate protease have a molecular weight of about 38,000, the molecule consists of single peptide chain and contains about 6% carbohydrate, the enzyme is stable at 38 °C and pH values 3-6 with optimum pH 4.5 for both stability and activity for denudated hemoglobin (Kazemi-Vaysari et al., 2002).

In general fermentation carried out by living cells can be using two types of fermentation, solid-state fermentation (SSF) and liquid state fermentation (LSF) or submerged fermentation (SF).

Submerged fermentations are usually carried out with a substrate which is either dissolved or remains suspended in an aqueous medium, and is suited for microorganisms such as bacteria that require high moisture (Subramaniyam, 2012), it consume substrate quickly so they need to add and replace substrate on an ongoing basis, it is expensive and needs more complex equipment.

Solid-state (substrate) fermentation (SSF) has been defined as the fermentation process occurring in the absence or near-absence of free water. SSF has higher productivity compared to (SmF), using of raw materials and agro-industrial residues

solves the pollution problem and reduce material cost. The lower water activity of the fermentation medium reduces the contamination risk especially by bacteria and yeast. Capital cost, energy expenditure and cost of downstream process are lower than SmF (Perez-Guerra et al., 2003; Krishna, 2005).

Consume the substrate slowly and gradually so it can be used for a long period of fermentation, do not need continuously mixing to bring homogeneity, ventilation processes simple, it does not require sophisticated technology, which need less energy and occupy less space (Al-Khafaji, 1990).

These advantages have made this system favorable in production of exoenzyme,mono-cell proteins, fermented foodstuff, toxins, organic acids, and some industrial chemicals, invertase, cellulose, lactase, aminoglycosides, pectinase and fungal protease.

In biotechnological processes, natural raw material, or recyclable waste material such as lignin, bran, wheat flour, rice flour, cotton, yeast extract, soy powder, beet molasses, starch, and cellulose are widely used as substrate. Wheat bran is a good choice for industrial production of enzymes. It is contains 65% carbohydrate, 16% protein, phosphate, calcium, iron, copper, magnesium, phosphate, potassium, sodium, zing, chlorine, lipids and vitamins B1, B2, B3, E, K (USDA, 2015) and is exclusively suitable for use in production of fungal enzymes (Singhania et al., 2010).

Some of the nutrients may be available in sub-optimal concentrations, or even absent in the substrates. In such cases, it would become necessary to supplement them externally with these.

The a viability of nitrogen in sufficient quantities is necessary to achieve the rapid growth of microorganism in all phases of growth, 15% of the dry mass of mycelium in filamentous fungi consists of nitrogen (Corbett, 1980), molasses, corn steep liquor, whey powder, soy flour, and yeast extract uses as raw materials rich in nitrogen added to the fermentation medium (Papagiani, 2004).

In this paper we studied the effect of supplying fermentation medium with deferent nitrogen resources; Urea, peptone, albumin, casein, yeast extract on biosynthesis of protease by fungi *Rhizomucor miehei* under solid state fermentation.

Materials and methods

Microorganism: Fungal strain in this study was *Rhizomucor miehei* ECC 841 (NRRL 3420) was obtained from Cairo MIRCEN - Faculty of Agriculture, Ain Shams University. It was maintained on (PDA) slants and stored in refrigerator at 4 °C for further use.

Inoculation medium: molds were inoculated in roux flask contains 100 mL (PDA), and incubate for 4 days at 25-30 °C, the inoculum prepared by scratching with the existence of 200 mL sterilized distilled water. Concentration of spore suspension was determined by counting on an advanced Neubauer Counting chamber and then was used for the inoculation of the culture medium.

Fermentation medium: wheat bran was used as base material, acidic salt mineral solution (100 mL) was preparing containing in (g/L); $ZnSO_4.7H_2O$: 0.07 : $MgSO_4.7H_2O$: 0.07 : $CuSO_4.7H_2O$: 0.07 : $FeSO_4$: 0.09; 0.2 N HCl, 10 mL of this solution was distilled to 1 L and 60 mL of it was used to moisten 100 g of wheat bran (Thakur, 1990), 20 g of moist wheat bran was distributed in 250 mL Erlenmeyer flask, and autoclaved for 20 min at 121 °C. After cooling flasks were inoculated with spore suspension with ratio 6_{10} /g. and incubate at 40 °C, for 4 days.

Optimizing nitrogen source: Urea, peptone, albumin, casein, yeast extract were added with concentrations (1%-10%), to determine the optimal source and concentration.

Enzyme extraction: After incubation period, the 100 mL of distilled water were added under shaking at 200 rpm

for 1 h at 4-10 ºC. The extract were filtered through (what man paper no. 1), the filtrate obtained was centrifuged at 6000 rpm for 20 min at 4 °C. The supernatant was used as crude enzyme source.

Protein content: was determined according to the method of Lowry, 1951).

Enzyme activity.

Milk clotting activity (MCA): MCA was determined according to the method of Arema et al. (1970).

Proteolytic activity (PA): was determined according to the method of Kunitz (1947).

Results and discussion

The main role of protease production is to degradation of complex proteins to simple composition protein easy consumed through cell (Rao et al., 1998).

The importance of nitrogen source showed in Figures 1 and 2, casein has increased enzyme production, the enzyme yield and enzyme activity has increased with adding casein and the higher value was at the concentration of 2%, adding casein with higher concentrations resulted in decreased in both protein content and enzyme activity.

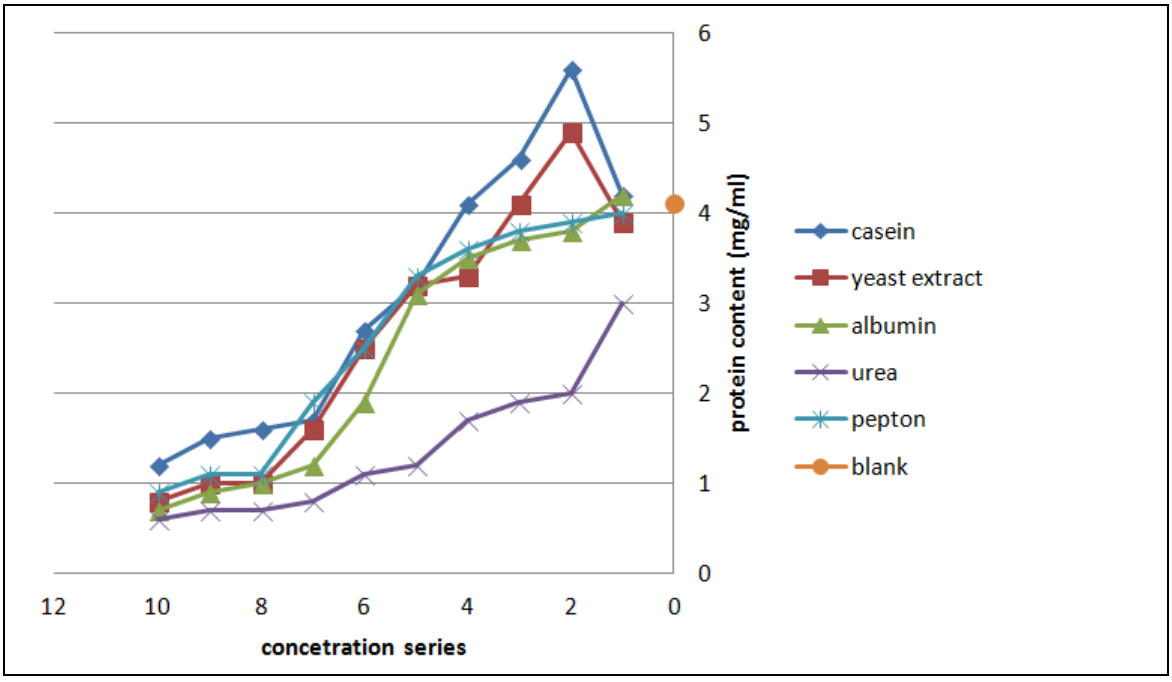

Figure 1. The effect of nitrogen concetration on protease production.

Fungi *Rhizomucor miehei* can exploit various nitrogen sources for the production of protease, addition of yeast extract up to 2% also increased protein content and enzymatic activity, addition yeast extract in (2%-4%) rates did not cause any increase in the yield or activity of the enzymes.

The continuing addition led to a decrease in both content and enzymatic activity and reached the lowest value at the rate 10%.

As shown in Figures 1 and 2, albumin and peptone has less effect on protease production and the enzyme activity.

When urea were added to the medium, the enzyme yield and the enzyme activity were lower than the control.

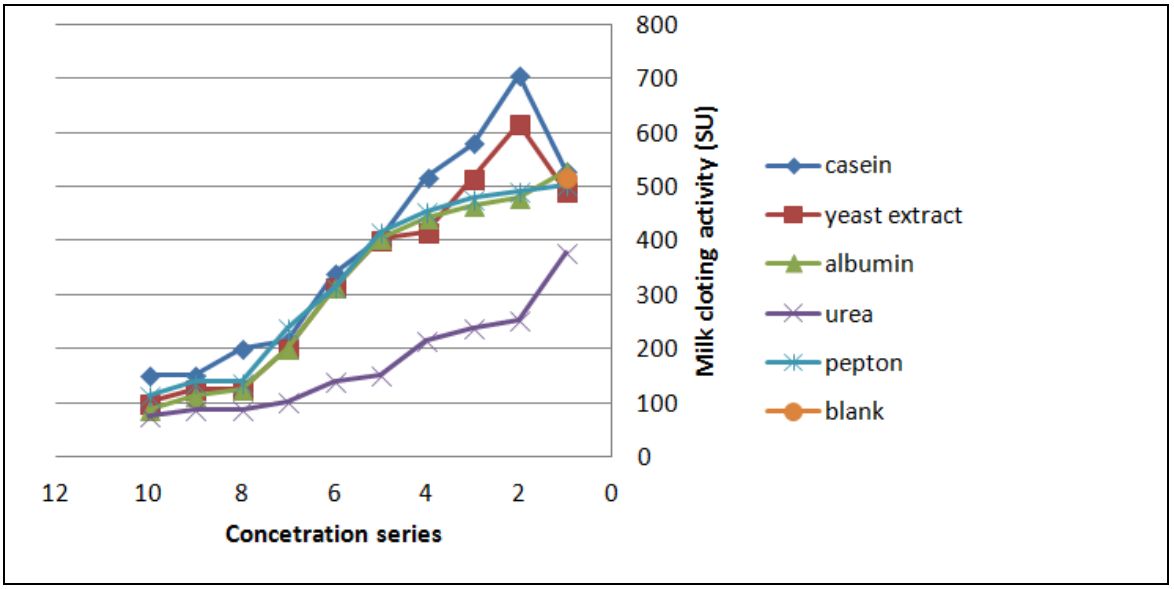

Figure 2. Effect of nitrogen source on milk cloting activity.

The variation in the production of protease according to the various nitrogen sources may be due to several reasons, including the difference in the physical and chemical nature of the nitrogen source, and it is one of the factors affecting microorganism growth and producing enzymes, especially in mediums with heterogeneous nature such as wheat bran, and the most important physical factors which is the surface area exposed to the microorganism reaction and material porosity and size of substrates (Nigam and Singh, 1994).

The chemical factors affecting the production of proteases is the nature of the of the constituent materials of the nitrogen source, whether simple or complex, in addition to the difference in the type and proportions of organic material in the source especially nitrogen, casein contains nitrogen by 15.9% (USDA, 2015), yeast extract contains nitrogen by 11% (Sigma Aldrich data base), peptone contains 10% nitrogen (Kurbanoğlu and Algur, 2002). Khademi et al. (2013) referred that inorganic sources has no effect on enzyme yield. Or is likely to fungi *Rhizomucor miehei* produces several types of protease that nitrogen source can stimulates.

The reason for the low secretion and enzymatic activity as a result of increasing the concentration of nitrogen source may explains that Encoded genes necessary for nitrogen consumption enzymes normally regulated by mechanisms of stimulation or specialized induction are subject to major control mechanism known (nitrogen metabolite repression). According to this mechanism genes expressed at high levels at the demand conditions of nitrogen only, but with the existence of ready and preferred sources of nitrogen it leads to give a signal to stop gene expression of catabolic enzymes (Marzluf, 1997).

Thus can keeping most closely associated nitrogen sources even preferred or available sources consumption (Berger et al., 2008).

Larcher et al. (1996) illustrate the reduction in protease production by *Scedosporium apiospermum* with addition of high concentrations of peptone that the free amino acids released by hydrolysis of this high quantity of peptone may cause enzyme repression.

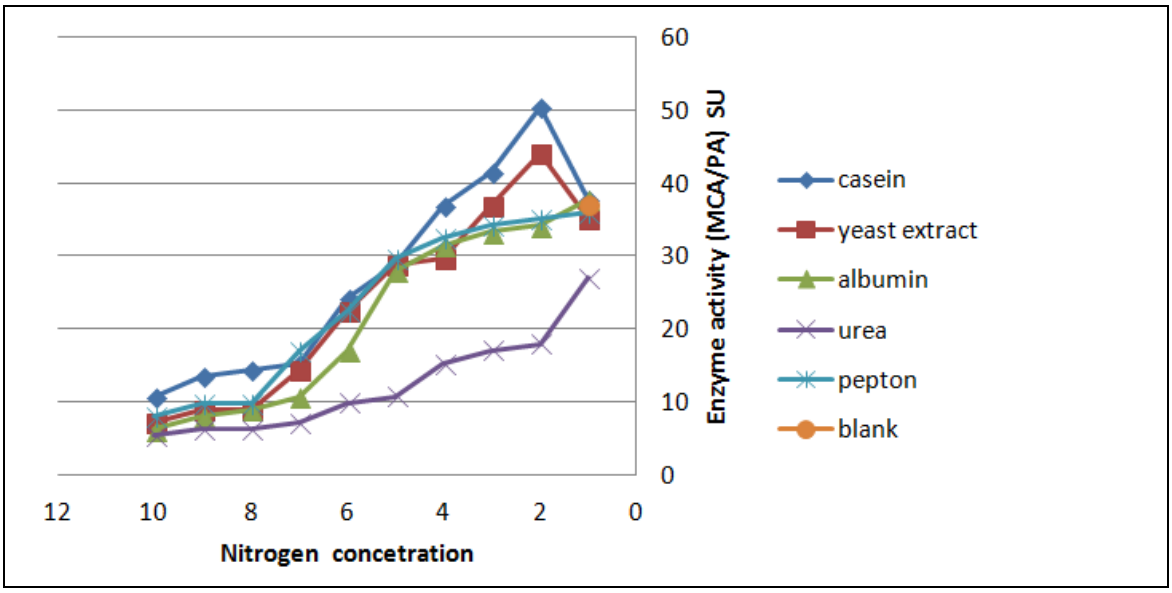

Figure3. The efect of nitrogen source on enzyme activity.

It seems that this phenomenon is widespread in fungi, especially filamentous fungi, as in the genus *Aspergillus* (Bouchara et al., 1993) and *Rhizopus* (Schindler et al., 1983).

Similar findings have been reported by other workers (Silveira et al., 2005; Foda et al., 2013; Khademi et al., 2013).

Conflict of interest statement

Authors declare that they have no conflict of interests.

Conclusion

Nitrogen has an important role in protease production, casein showed to be the best source of nitrogen, supplement medium with 2% (w/w) casein lead to the highest enzyme yield and enzyme activity with values (5.6 mg/mL, 50.5 SU), respectively, compared to 4.9 mg/mL, 44.1 SU, 4.2 mg/mL, 37.8 SU, 4 mg/mL, 36 SU, 3mg/mL, 27 SU, for 2% yeast extract, 1% albumin, 1% peptone, and 1% urea, respectively.

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