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Original Article

Study the influence of culture conditions on rennin production by *Rhizomucor miehei* using solid-state fermentations

Houthail Alahmad Aljammas*, Hassan Al Fathi, Walid Alkhalaf

Department of Food Science, Agriculture Engineering Faculty, Al-Furat University, Deirazzor, Syria

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ABSTRACT

Investigations were conducted on the production of Rennin enzyme from the fungi *Rhizomucor miehei* 3420 NRRL using Solid-State fermentation. Wheat bran was used as a substrate. The influence of moisture content, incubation temperature, and the initial pH of fermentation medium were studied. The protein content, milk clotting activity (MCA), specific activity, proteolytic activity (PA), and (MCA/PA) ratio of the extracted enzyme were calculated after 4 days of incubation to evaluate the quality of the enzyme. The results showed that the optimal conditions for production were as follows: incubation temperature of 40 °C, moisture content of 60%, and pH of (3). Under these conditions, a production process of Rennin enzyme was established, and the values of protein content, milk clotting activity, specific activity, proteolytic activity, and (MCA/PA) ratio reached to 4 mg/mL, 600 SU/mL, 150 SU/mg, 45 PU/mL, 13.3 respectively.

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1. Introduction

Rennin enzyme (chymosin) is used in the manufacture of cheese, it is also known as acidic protease. The coagulation of milk by rennin takes place in two stages: During the first stage or the enzymatic phase, casein is hydrolyzed by rennin, thus releasing of para-casein which form the curd in the second stage (non-enzymatic phase) [17].

Rennet is extracted from the fourth stomach of an un weaned calves, and it's purified active components are called rennin.

The world wide increase in cheese production coupled with reduced supply of calf rennet have led to an increase in the demand for alternative source of milk coagulants.

Animal and plant coagulants have been used as rennet substitutes, but these have showed unsatisfactory properties, in addition to microbial coagulants which have gained wide acceptance.

Extensive researches have been carried out so far on some species of bacteria and fungi like *Aspergillus oryzae*, *Irpeslactis* [15,8,16], *Rhizopus* sp. [26,7].

Many Microorganisms are known to produce proteases, but it's coagulants is not suitable as substitutes for calf rennet.

The bacterial proteases have proved to be unsuitable because of its high nonspecific proteolysis activity. Recently studies have focused on three fungal strains to produce microbial rennet namely *Rhizomucor miehei*, *Endothia parastica*, *Rhizomucor pussilus* [27,10,23,11].

The protease of *Rhizomucor miehei* is the preferred substitute for calf rennet because of its specificity in splitting of peptide bonds in kappa-casein similar to calf rennet [5], high ratio of milk coagulating activity, identical calcium requirements, good cheese quality [10] and lower incidence of bitter flavor in cheese curd [28].

In general, two types of fermentation systems are used in biotechnology processes: solid-state fermentation (SSF) and liquid state fermentation (LSF).

Submerged fermentation (SmF) or liquid State fermentation (LSF) are usually carried out with a substrate which is either dissolved or remains suspended in an aqueous medium, and it is suited for microorganisms such as bacteria that require high moisture [25], it is expensive and requires more complex equipment. Substrate is quickly consumed so there is need to add and replace it constantly.

Solid-state fermentation (SSF) has been defined as the fermentation process occurring in the absence or near-absence of free water.

SSF has a higher productivity compared to (SmF), use of raw materials and agro-industrial residues solves the pollution problem and reduce material cost. The lower water activity of the fer-

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* Corresponding author.

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mentation medium reduces the contamination risk especially by bacteria and yeast. Capital cost, energy expenditure and cost of downstream process are lower than SmF [20,12].

Substrates are consumed very slowly and constantly. Hence, there is no need to supply the substrate for longer time. SSF does not require highly advanced technology, the size of reactor is small, simply in ventilation systems, consuming less energy, and occupy less space [2].

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 contains 65% carbohydrate, 16% protein, phosphate, calcium, iron, copper, magnesium, phosphate, potassium, sodium, zinc, chlorine, lipids and vitamins B1, B2, B3, E, K [30] and it is exclusively suitable for use in production of fungal enzymes [24].

Despite the widespread use of solid-state fermentation, Not enough information is available on kinetics of reactions in SSF systems. This is mainly because of difficulties involved in the measurements of growth parameters, analysis of cellular growth and determination of substrate consumption, etc., which is caused due to heterogeneous nature of the substrate, which are structurally and nutritionally complex [19]. In this paper we studied the influence of culture conditions on protease biosynthesis by *Rhizomucor miehei* under solid state fermentation to optimize rennin production process, and we have investigated the interactions in SSF systems.

2. Materials and methods

Fungal strain: *Rhizomucor miehei* EMCC 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 from slants were used to inoculate roux flasks containing 100 mL of (PDA). The inoculated flasks were incubated at 37 °C for 4 days. The inoculum was obtained by scraping the PDA surface in the present 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 fermentation medium.

Fermentation medium: wheat bran was used as the base material. Acidic mineral salt solution was prepared of the composition (g/L); ZnSO₄·7H₂O: 0.07, MgSO₄·7H₂O: 0.07, CuSO₄·7H₂O: 0.07, FeSO₄: 0.09; 0.2 N HCl. 10 mL of this solution was diluted to 1 L [27]. Appropriate volume of this final solution was added to 100 g of wheat bran to obtain the desired initial moisture content, 20 g of the moist wheat bran was distributed in 250 mL Erlenmeyer flasks, and autoclaved for 20 min at 121 °C. After cooling, the flasks were inoculated with the spore suspension with the ratio of 10⁶ spores/g. The cultivation was carried out at different temperatures for 4 days [1].

2.1. Optimization of Cultural parameters

Effect of moisture content: different volumes of the mineral solution were added in order to obtain the moisture content values of (10–80% v/w).

Effect of temperature: To investigate the effect of temperature, fermentation was conducted at the temperatures of (30–50 °C).

Effect of initial pH: appropriate volumes of HCl solution were added to reach to different initial pH of the fermentation medium (2, 2.5, 3), (including the moistening solution to maintain the desirable moisture content).

Enzyme extraction: After incubation period, 100 mL of distilled water were added to the solid fermentation medium. The content was shaken at 200 rpm for 1 h at 4–10 °C. The extract were filtered through (whatman 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 et al. [14].

2.2. Enzyme activity

Milk clotting activity (MCA): MCA was determined according to the method of [3] and expressed in terms of Soxhlet Units.

(S.U.). One Soxhlet unit is defined as the quantity of enzyme required to clot one mL of substrate containing 0.1 g skimmed milk powder and 0.0014 g calcium chloride in 40 min at 37 °C and was calculated with the formula: unit of milk-clotting activity.(U) = 2400/T * S/E where T is the time necessary for clot formation, S is the milk volume and E is the enzyme volume.

Proteolytic activity (PA): was determined according to the method of [13] using casein as a substrate. One mL of 1% casein in 0.1 M (Sorensen buffer) (pH 6.7) and 1 mL of enzyme were incubated at 35 °C for 20 min. The reaction was terminated by addition of 3 mL of 5% trichloro acetic acid solution and was centrifuged at 5000 rpm for 10 min at 10 °C. Absorbance was measured at 280 nm. One unit of protease activity was defined as the activity which gives rise, under the conditions described, to an increase of one unit of optical density at 280 nm per minute digestion.

2.3. Experimental design and statistical analysis

The experiments were designed by changing one factor at a time by three replicates per treatment and averaged. All the results were analyzed using analysis of variance test (ANOVA) and the least significant difference (LSD) at a significant level of 0.01 using IBM SPSS Statics 21.

3. Results and discussion

3.1. Moisture content effect

After inoculation, the flasks were incubated at 37 °C for 4 days at different moisture content (10–80% v/w) with 10% intervals, and the protease production was studied for each treatment.

At the moisture content of 30%, the enzyme excretion was low and the enzyme activity was negligible, the moisture content at this level probably is not enough for the fungal growth, This could be explained by The low solubility of nutrients in the solid substrates, a lower degree of substrates swelling, and higher water tension.

The protein content and enzyme activity was affected by increasing in moisture content up to 40% significantly, and there were no significant differences at 50% except proteolytic activity, but with increasing in moisture content up to 60% there was a significant difference at all indicators and the maximum enzyme production was observed at this level. High moisture content may lead to increase in nutrient solubility, also the absorption of water by wheat bran particles lead to swallowing which increase in its surface area.

The higher moisture content levels (70–80%) led to reduction in the enzyme production, This could be explained by the decrease in porosity, loss of particulate structure, development of stickiness, reduction in gas volume, decreased gas exchange and enhanced formation of aerial mycelium [18].

Table 1

The effect of moisture content on enzyme production.

Moisture content (%)	Protein content mg/mL	Milk clotting activity SU/mL	Specific activity SU/mg	Proteolytic activity PU/mL	MCA/PA
30	0.42a	10a	23.8	12.3	0.8a
40	2.5b	380b	152a	72	5.2bc
50	3.1bc	441b	140ab	56.8	7.7b
60	3.8c	550	144.7ab	41.6	13.2
70	1a	131	131b	28.35	4.6bc
80	0.2a	20a	50	2.8	3.5ac
LSD _{0.01}	0.88	107.19	13.66	7.8	2.97

*Within columns, means followed by the same letter(s) are not significantly different at the 0.01 level.

Table 2

The effect of incubation temperature on enzyme production.

Temperature (°C)	Protein content mg/mL	Milk clotting activity SU/mL	Specific activity SU/mg	Proteolytic activity PU/mL	MCA/PA
25	0	0	0	0	0
30	1.8a	125a	69.4	35a	3.5a
35	2.7ab	255b	94.4a	41a	6.2b
40	4c	600	150b	45a	13.3
45	3.1bc	446	143b	40a	11.1
50	2a	190ab	95a	35a	5.4ab
LSD _{0.01}	0.96	96.47	22.68	11.38	2.04

*Within columns, means followed by the same letter(s) are not significantly different at the 0.01 level.

Table 3

The effect of initial pH on enzyme production.

Initial pH	Protein content mg/mL	Milk clotting activity SU/mL	Specific activity SU/mg	Proteolytic activity PU/mL	MCA/PA
6 (blank)	3.2ab	469	146.5a	36ab	13a
2.5	2.5b	164.7 a	65.8b	24b	6.8b
3	4a	600	150a	45 a	13.3a
3.5	3ab	274 a	91.3b	30b	9.1b
LSD _{0.01}	1.21	116.11	40.47	13.34	3.22

*Within columns, means followed by the same letter(s) are not significantly different at the 0.01 level.

Similar findings have been reported by other workers [27,21,9] (see Table 1).

3.2. Effect of incubation temperature

fermentation was conducted at different temperatures (30–50 °C) with 5 degrees intervals, medium was moistened with ratio of 60%, and incubated for 4 days.

The optimal temperature for the enzyme production was 40 °C, lower and higher temperature affected the protein content and enzyme activity. Because of the impact on growth and enzymatic reactions within the living cell, which is reflected on the synthesis of enzymes.

No enzyme synthesis was observed at 25 °C, This was probably due to the lack of growth of the inoculated fungal spores. The significant differences started to appear at 30 °C, and there was gradually increase in enzyme production coupled with temperature increase. The maximum protein content and enzyme activity was reached at a temperature of 40 °C. The enzyme synthesis started to decrease at 45 °C and there was a sharp decrease in the protein content and enzyme activity at 50 °C, that was probably due to the generation of excess heat that denatured the enzyme or evaporated the moisture of the medium.

Shumi et al. [22] stated that *Fusarium tumidum* proteases are thermo labile and show reduced activities at high temperatures. Conn et al. [4] found out that High temperature have some adverse effects on metabolic activities of microorganism and cause inhibition of the growth of the 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 [29].

Similar findings have been reported by [27,10,6] (see Table 2).

3.3. Effect of initial pH

HCl solution was used to adjust the initial pH of fermentation medium to (2, 2.5, 3), blank had no addition of acidic solution (pH 6). Moistening ratio was (60% v/w) and the fermentation carried out at 40 °C for 4 days.

The protein content and enzyme activity was low at (pH 2.5). (MCA), specific activity, and (MCA/PA) were affected significantly, due to the reduction of the pH and thus the lack of nutrient solubility, and instability of enzymes toward pH.

The Highest protein content and enzyme activity was obtained at (pH 3), this could be explained by the effect of pH on nutrient solubility, its ionization, and its viability to move into the cells.

When pH was (3.5) all the indicator were affected negatively, showing that a possible catabolic repression occurred because of the free carbohydrate from wheat bran hydrolysis [27].

Similar findings have been reported by Thakur et al., Silveira et al. [27,23] (see Table 3).

4. Conclusion

It should take into consideration the critical factors which have a significant effect on the enzymatic production process such as temperature, moisture content, and pH.

The optimal conditions for rennin production by *Rhizomucor miehei* using SSF in this study represented in incubation at 40 °C, humidity ratio of 60%, and pH of 3.

Milk clotting enzyme produced under optimized conditions has values of 4 mg/mL, 600 SU/mL, 150 SU/mg, 45 PU/mL, 13.3 for protein content, milk clotting activity, specific activity, proteolytic activity, and (MCA/PA) ratio respectively. This shows the higher enzyme productivity of solid-state fermentation.

Higher milk clotting activity and lower proteolytic activity shows that the proteases produced under the above-mentioned conditions suitable for the manufacture of cheese, and can be considered as a suitable substitute for calf rennet.

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