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Production of Milk Clotting Enzyme from Aspergillus oryzae under Solid-State Fermentation using Mixture of Wheat Bran and Rice Bran

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Abstract- Microbial rennet-like milk-clotting enzymes are aspartic proteinases that catalyze milk coagulation, substituting calf rennet. Crude enzymatic extract produced by the Aspergillus oryzae NCIM 1032, on solid state fermentation (SSF) using mixture of wheat bran and rice bran (7:3), exhibited high milk-clotting activity (MCA) and low proteolytic activity (PA) after 120 h of fermentation. Highest milk-clotting activity was at pH 7.5, at 30 ºC. Glycerol (5%) was found to be best solvent for leaching out milk clotting enzyme. The yield of enzyme was improved with the supplementation of glucose and beef extract as a carbon and nitrogen source respectively. Metabolic heat generated due to fermentation was equally distributed throughout the substrate bed by agitation in rotating drum bioreactor and enzyme production increased at speed of 25 rpm and at intermittent agitation (1 min/day). High ratio of milk clotting to proteolytic activity strengthens the potential usefulness of milk-clotting enzyme of Aspergillus oryzae NCIM 1032 as a substitute for calf rennet in cheese manufacturing.

Index Terms- Solid state fermentation, Milk Clotting activity, Proteolytic activity, *Aspergillus oryzae,* Rotating drum bioreactor, Calf rennet

I. INTRODUCTION

Rennet (EC 3.4.23.4), an aspartate protease, is the main enzyme employed in cheese production. Rennet not only clots the milk but also plays an important role during cheese maturation, which is a vital and complex process for the balanced development of flavor and texture [1] .Traditionally natural calf rennet is used for the production of cheese which is extracted from the inner mucosa of the fourth stomach chamber (The abomasum) of slaughtered young, unweaned calves. These stomachs are the by product of veal production. Rennet is a complex enzyme and contains mixture of enzymes, including a proteolytic enzyme (Protease) that coagulates the milk. The active enzyme in rennet is called chymosin or rennin. For developing country like India slaughtering of unweaned calves or even young calves to get abomosa solely for rennet extraction is seen as a very expensive means. As a cheaper alternative if rennet is extracted from older calves the rennet contains less or no chymosin but a high level of pepsin. High pepsin content in animal rennet hydrolyses proteins which weakens the protein network in the milk. This results in lower cheese yield with reduced protein and fat content and bitter flavor.

A worldwide shortage of aspartate protease-chymosin, (EC 3.4.23.4), a natural calf rennet, traditionally used in the production of cheese, has prompted a search for an adequate substitute. Microbial enzymes are especially favored since they can be mass cultured and offer a variety of properties permitting selection of those most suitable in cheese production. Fungal and bacterial sources are widely used for cheese production. In the USA alone, about 60% of the cheese is manufactured using fungal enzymes [2]. Comprehensive studies that have been carried out on rennin like enzymes of microbial origin, aimed at their utilization as rennin substitutes for cheese making [3]. The production of a milk-clotting enzyme was reported in *Penicillium oxalicum* [4] and *Nocardiopsis* sp. [5]. In the case of microbial rennets from *Mucor* sp., none of the available reports contains any indication that the use of the enzyme in cheese manufacture is unsafe [6]. An adequate substitute must have intense milk clotting and low proteolytic activities to minimize dissolution of the clot [7].

Solid substrate fermentation (SSF) has been known for centuries and used successfully for the production of oriental foods [8]. In recent years, SSF has shown much promise in the development of bioprocesses and products [9, 10]. More recently, it has gained importance in the production of microbial enzymes due to several economic advantages over conventional submerged fermentation (SmF). Several reports on SSF have been published on the production of fine chemicals [11-13], enzymes [14,15], antibiotics [16,17], and immunosuppressant [18,19]. SSF processes are therefore of special economic interest for countries with abundance of biomass and agro-industrial residues, as they can be used as cheap raw materials. In this process, the solid substrate not only supplies the nutrient to the culture but also serves as an anchorage for microbial cells. Cost and availability are important considerations, and therefore the selection of an appropriate solid substrate plays an important role in the development of efficient SSF processes. SSF has generated much interest because it offers lower manufacturing costs by utilizing unprocessed or moderately processed materials. SSF is generally a simpler process and requires less preprocessing energy than SmF (Submerged fermentation). Further, the initial capital costs are less for SSF. Other advantages are superior productivity, low waste water output, and improved product recovery [20]. However this technique shows several disadvantages over submerged fermentation , which have discouraged its use for industrial production[21]. The low moisture and poor thermal conductivity of the substrate make heat transfer and temperature control difficult in SSF [22]. Many bioreactors

have been traditionally used in SSF processes. These can be mainly classified in two groups: the ones which show an agitation system and the ones which work in static conditions. The first category comprises rotating drums, gas-solid fluidized beds, rocking drums, horizontal paddle mixer, etc, while the second one includes the packed-bed and the trays bioreactor. Static beds are required when the substrate bed must remain static throughout the growth phase or when the substrate particles have to be knitted together by the fungal mycelium[23] . On the other hand, the use of mixed bioreactors improves the homogeneity of the bed and ensures an effective heat and mass transfer [35]. However, the shear forces caused by rotation and agitation damage or disrupt fungal mycelia and reduce the porosity of the substrates [24].

II. Research Elaborations

2.1) Micro organism and growth:

Fungal strain Aspergillus oryzae was purchased from NCL and maintained on potato dextrose agar slants at 4ºC.

2.2) Raw Material:

Rice bran, Wheat bran , Jowar Bran, pigeon pea husk, chickpea husk, groundnut meal was purchased from local market. Raw material were properly dried at 60ºC.

2.3) Inoculum preparation:

Sporulated culture lawn of the *Aspegillus oryzae* was picked up and mixed well in sterilized distilled water. The spore suspension will be diluted to a concentration of 10^8 spores/ml and used in inoculum.

2.4) Milk clotting enzyme assays:

2.4.1) Assay for milk clotting activity:

Milk-clotting activity (MC) will be assayed by a modified procedure of Arima et al. (1970) [25] : 1 ml substrate (8.4% Skim milk powder in 0.05 M sodium acetate buffer, pH 5.3, containing 0.01M $CaCl_2$) will be mixed with 0.1 ml enzyme and the clotting time will be measured and expressed in **Soxhlet units. One Soxhlet unit is defined as the quantity of enzyme required to clot one ml of substrate in 40 min at 37°C.**and was calculated according to Shata [26]: 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.

2.4.2) Assay for Proteolytic activity (PA):

The substrate employed for the assay of proteolytic activity will be soluble casein . The casein solution will be prepared by the method of Kunitz (1947) [27]:
a) 1 ml of diluted enzyme solution will be added to 2 ml of casein solution (pH
6.0) and 1 ml of 1.0 M phosphate buffer (pH 6.0); the resultant solution will be mixed well and kept at 40ºC for 20 min.
b) Then, 5 ml of 5 % trichloroacetic acid will be added, and the mixture allowed to stand for 30 min at room temperature.
c) The resultant precipitate will be removed by filtering the solution through Whatman no. 4 filter paper. The concentration of split products in the filtrate will be determined essentially by the method of Layne (1957) [28].
d) To 2 ml of filtrate, 4 ml of alkaline copper sulfate solution and 0.4 ml of distilled water will be added.
e) After 10 min, 0.1 ml of diluted Folin's reagent will be added, and the mixture Will be held for 30 min or longer for development of color.
f) The optical density will be read at 750 nm in a spectrophotometer . Optical density expresses enzyme activity.

2.5) Production of MCE under SSF:

Wheat bran (10 gm) were taken in 250 ml conical flasks and moistened with 10 ml of mineral salt solution (g/l : 2.0, KNO_3; 0.5, $MgSO_4 \cdot 7H_2O$; 1.0, K_2HPO_4; 0.439, $ZnSO_4 \cdot 7H_2O$; 1.116, $FeSO_4 \cdot 7H_2O$; 0.203, $MnSO_4 \cdot 7H_2O$; and pH 7.0). the medium were autoclaved at 121.5ºC for 20 min and after cooling inoculated with 1 ml of spores suspension and incubated at 30ºC for a weak. Same procedure was follow for all other substrates.

2.6) Enzyme extraction:

After 120 hr of incubation a known quantity (2 gm) of solid medium was extracted with distilled water by shaking on a rotary shaker (220 rpm, 1hr, 30ºC). The filtrate obtained was centrifuged at 10,000 g for 10 min at 4ºC. The supernatant was used as crude enzyme source for milk clotting protease activity.

2.7) Effect of substrates:

Different substrates like wheat bran, rice bran etc were tried for enzyme production. Then two highest enzyme producing substrates were selected from above mentioned substrates and were mixed in different combinations such as 1 gm Wheat bran and 9 gm Rice bran, 2 gm Wheat bran and 8gm of Rice bran up to 9 gm Wheat bran and 1gm Rice bran. Enzyme activity was monitored at 24 hrs interval up to 168 hrs. The highest enzyme producing combination of substrates were used in SSF for further optimization.

2.8) Effect of incubation time:

After inoculation, the flasks were incubated at 30ºC for different time periods ranging from 24 hrs to 168 hrs and enzyme activity was monitored.

2.9) Effect of incubation temperature:

The SSF was carried out at different temperatures such as 30 ºC,40 ºC,50 ºC,60 ºC and70 ºC for 120 hrs and the enzyme activity was assayed.

2.10) Effect of pH of moisturizing agent:

SSF was carried out using moisturizing agent with different pH ranging from 3.5 to 8.5. the flasks were incubated at 30 ºC for 120 hrs and the enzyme production was measured as described earlier.

2.11) Effect of initial moisture content:

The substrate was moistened using D/W in different ratios (w/v) starting from 1:1, 1:2, 1:3, 1:4 to find out the best ratio for enzyme production under SSF.

2.12) Effect of carbon and nitrogen source supplementation:

The SSF production medium was supplemented with different C and N sources like Glucose, sucrose etc supplemented and the flasks were incubated at 30 ºC for 120 hrs.

2.13) Effect of organic solvent on extraction:

After cultivation, a known quantity of solid medium were extracted with distilled water and various organic solvents like Glycerol , Sorbitol, ethanol, methanol in concentration of 5% (1:10 v/w) by shaking on a rotary shaker (220 rpm, 1 h, 30ºC). The filtrate obtained was centrifuged at 10,000 g for 10 min at 4ºC. The supernatant was used as crude enzyme source for milk-clotting protease activity.

2.14) Effect of agitation :

Rotating drum Bioreactor:

The lab scale fermentor consist of 250 ml glass roller bottle. For the experiment carried out in agitation the bottle was place in roller system composed of two cylinders which rotates continuously in this way the media of fermentor was mixed as a consequence of the movement of the bottle.

2.14.1) Agitation speed: For the fermentation 10 gm of preinoculated solid substrate were added to bottle . the substrate bed leaving enough space to obtain good agitation. The system was incubated at 30 ºC for 120 hr. the effect of agitation time and agitation speed in the production of milk clotting enzyme were evaluated in rotating drum bioreactor. For this purpose intermittent agitation (one agitation of 2 min/ day) were carried out at different speeds such as 25 rpm,50 rpm ,75 rpm, 100 rpm,125 rpm.

2.14.2) Agitation time: to optimized agitation time the bottles were agitated at constant optimized speed from 1 min/ day up to 5 min/day. The milk clotting enzyme production was monitored at the end of 120 hr.

III. Results

3.1) Substrate

Milk clotting enzyme activity of each substrate was calculated according to Shata [26]. Maximum milk clotting enzyme was observed in wheat bran (333.3 U/gm)followed by rice bran (322.6 U/gm)and other substrates showing less productivity. On the other hand these two substrates showing less proteolytic activity (Figure 2). So these two substrates were selected for combination.

3.2) Substrate combination:

Above two best selected substrates were mixed in different combination and flasks were incubated at 30 ºC and the enzyme activity was measured after different time periods. The enzyme production was maximum for combination of wheat bran and rice bran in the ratio of 7:3 (Figure 3). This combination was shows maximum milk clotting activity (513.1 U/gm) and less proteolytic activity (0.147). This combination was used for further optimization.

3.3) Incubation time:

After inoculation, the flasks were incubated at 30 ºC and the enzyme activity measured after different time periods. The Figure 4 shows enzyme production started after 24 hr of incubation and increased with time peaking at 120 hr (487.0 U/gm) there after , the enzyme production started decreasing and proteolytic activity was decreased up to 120 hr (0.163) and thereafter it started increasing.

3.4) Incubation temperature:

The SSF was carried out by incubating the flasks for 120 hr at different temperatures such as 30 ºC,40 ºC,50 ºC,60 ºC,70 ºC. The Figure 5 shows maximum enzyme production was observed at 30 ºC (532.5 U/gm) and proteolytic activity was 0.198 . Above 30 ºC there was decrease in the enzyme production.

3.5) Effect of pH :

SSF was carried out using nutrient solution as moisturizing agent with different pH ranging from 3.5 to 8.5 . Figure 6 shows maximum production of enzyme was observed at pH 7.5 (MCA : 516.4 U/gm and P.A: 0.156)

3.6) Initial moisture content:

Substrate was moistened using distilled water in different ratios (w/v) starting from 1:1, 1:2, 1:3 to 1:4. Figure 7 shows a ratio of 1:1 was found to be the best ((MCA : 532.5 U/gm and P.A: 0.142).

3.7) Effect of external Carbon and Nitrogen source:

The SSF production medium was supplemented with different Carbon and Nitrogen sources separately. Glucose and beef extract having positive results for production of enzyme (Table 1 &2). These sources was further studied to determine optimum concentration. 0.4% glucose and 0.8 % beef extract with supplementation of the medium there was a slight increase in enzyme production with carbon (546.3_U/gm)and nitrogen source 541.3(U/gm) (Figure 8 & Figure 9).

3.8) Effect of extractant:

5 % of glycerol solution shows approximate 96 % increase (1194.4 U/gm) in extraction than extraction by distilled water (Figure 10).

3.9) Effect of agitation speed:

The highest enzyme activities, obtained with speed of 25 rpm (612.7 U/gm) were assayed when the system was agitated 2 min/day (Figure 11). A high speed could damage fungal cultures as a consequence of the shear forces during mixing. Optimized agitation speed was 25 rpm.

3.10) Effect of agitation time:

The highest enzyme activities, obtained with agitation time of 1 min/day with the speed of 25 rpm (698.3 U/gm) (Figure 12). Agitation shows approximately 20 % of increase in production.

IV. Discussion

The selection of an ideal agroindustrial residues for enzyme production in SSF process depends upon several factors, mainly related with cost and avaibility of substrate material , and thus may involve screening of several agro industrial residue [29]. Various agroindustrial substrates were screened for the study. It was interesting to note that in SSF by *Aspergillus oryzae NCIM 1032* all the tested substrate showed enzyme production. Fermentation of wheat bran and rice bran showed maximum production of milk clotting enzyme among all substrates. There are several reports describing wheat bran as potent substrate for milk clotting enzyme production by *A. oryzae* [30], *Rhisomucor*[31], *Absedia ramose*[29]*and M. miehei*[32] in SSF. In another report mixture of rice bran , rice husk, and gram hull in the ratio of 5:3:2 was found to be suitable for protease production [33]. In this study maximum enzyme production (51.31 U/gm) was observed with mixture of wheat bran and rice bran in the ratio of 7:3. It may because of wheat barn is rich in proteins. It contains gluten , a viscoelastic protein. On the other hand rice bran has considerably lower protein content as compared with wheat barn and and is rich in tocopherols. It contains about 20 % of oil. So the mixture of wheat bran and rice bran satisfies more nutritional requirements than single substrate.it was interesting to note that , none of the reports was evidenced on the production of milk clotting enzyme using mixture of wheat bran and rice bran in the ratio of 7:3 as a substrate. In another study pigeon pea husk i.e dhal husk shows maximum production [34].

Maximum yield of milk clotting enzyme was obtained after 120 hr of incubation at 30 ºC. Decrease in production after 120 hr

of incubation may be due to the accumulation of ends products which hampers milk clotting enzyme production or may be due to the accumulation of toxic metabolites secreted during fermentation [35,36] or may be because of depletion of nutrients available to micro organisms . Similar findings shows by other workers [37,38].

The pH of the medium strongly affects many enzyme processes and transport of various compounds across the cell membrane [39]. In present study optimum pH for enzyme production was 7.5. similar results were reported by Yu and Chou[40] according to their results maximum enzyme production was at pH 7 by *Amylomyces rouxii*. D'souza and Perreira [41] have observed that milk clotting enzyme production by bacillus Licheniformis showed maximum production at pH 7.0.

R. Sathya and co-workers reported that optimum temperature of 30 ºC for milk clotting enzyme production by local isolate of *Mucor circinelloides* under SSF using dhal husk as substrate. With increase in temperature , sporulation is induced thereby hampering mycelia growth.

Milk clotting enzyme yield was maximum when substrate to water ratio was 1:1 (w/v) above this the enzyme production was found to be decreased. This could be explained by the fact that lower moisture levels lead to reduced solubility of the nutrients in the solid substrates a lower deree of substrate swelling and higher water tension. Similarly, higher moisture content were reported to caused decreased porosity, loss of particulate structure , development of stickiness , reduction in gas volume, decreased gas exchange and enhanced formation of aerial micellium [28]. Similar findings have been reported by other workers [42,43].

Among the various carbon sources tested glucose was found to be the best source for milk clotting enzyme production followed by maltose. Abdel- Fattach and Saleh [44] found that sucrose was optimum for the production of milk clotting enzyme from *Aspergillus versicolor.*

Supplementation of beef extract as a nitrogen source resulted in increase in enzyme production followed by peptone. The effect of specific nitrogen supplement on milk clotting enzyme production differ's from organism to organism , although the complex nitrogen sources are usually used for milk clotting enzyme production.

Agitation (1 min /day) shows approximate 20 % increase in production of enzyme. This may be because of metabolic heat produced within bed is regulated by agitation. Low moisture, poor thermal conductivity of the substrate result in poor heat transfer in SSF. Hence it is very difficult to maintain favorable temperature in the reactor. Thus water addition with intermittent mixing is favourable in SSF, which can be achived in rotating drum bioreactor. Water activity could also drop duu to build up of solutes such as glucose, amino acids etc. this could be prevented by spraying water on to the solid substrate coupled with mixing. The importance of evaporative cooling and moisture content of the substrate on the performance of SSF bioreactor has been heighted in the literature of Nagal and Lonsane [45,46] to control the rising temperature.

As speed of agitation increases production of enzyme was decreases because more speed could damage fungal cultures as a consequence of the shear forces during mixing.

V. Conclusion

High milk clotting and lower proteolytic activities were found in the enzymatic extract obtained after 120 hr of solid state fermentation by *A. oryzae* NCIM 1032 using mixture of wheat bran and rice bran in the ratio of 7:3 as substrate. In present study laboratory rotating drum bioreactor was designed and operated , the maximum enzyme activities were obtained in agitated condition at speed of 25 rpm (1 min /day). Best results were reached in low agitation speed and minimum duration. This agitation shows approximate 20% of increase in production.

VI. Figures

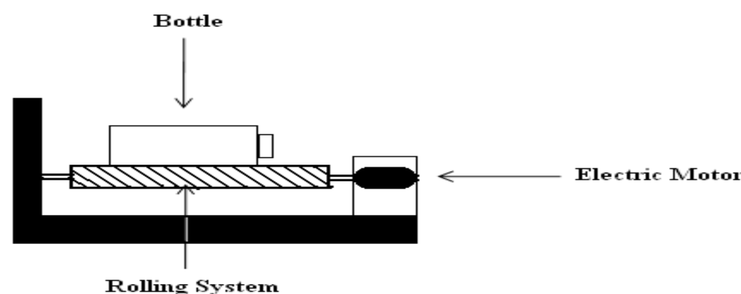

Figure 1: Laboratory scale rotating drum bioreactor.

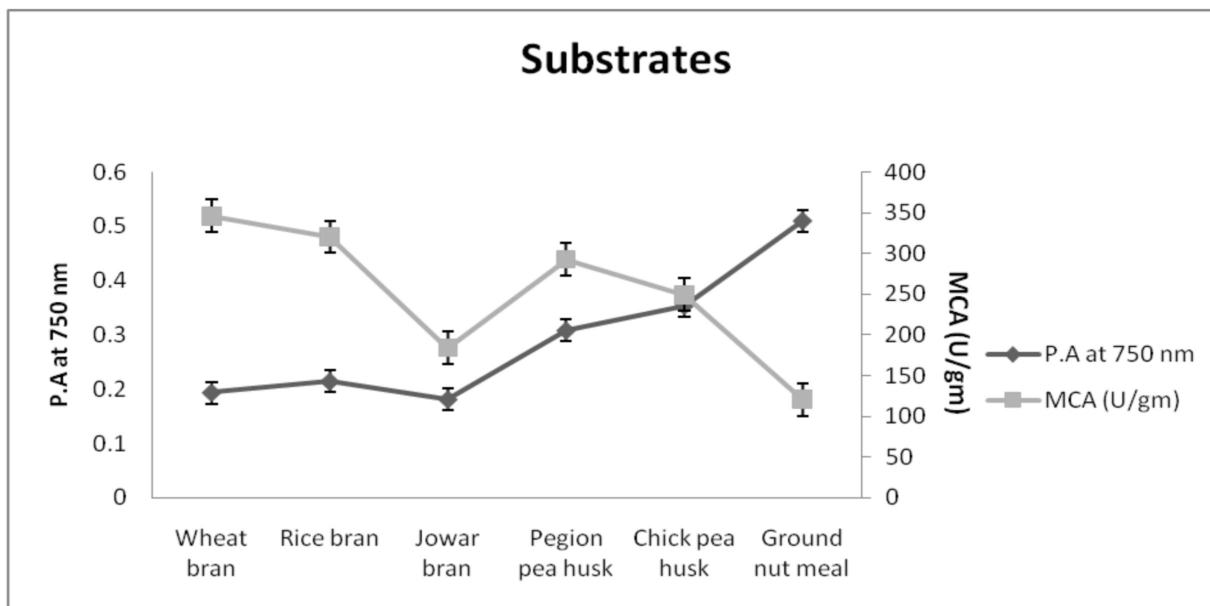

Figure 2: Production of milk clotting enzyme under SSF using different agri industrial wastes (Error bars represent standard deviations of three replicates.)

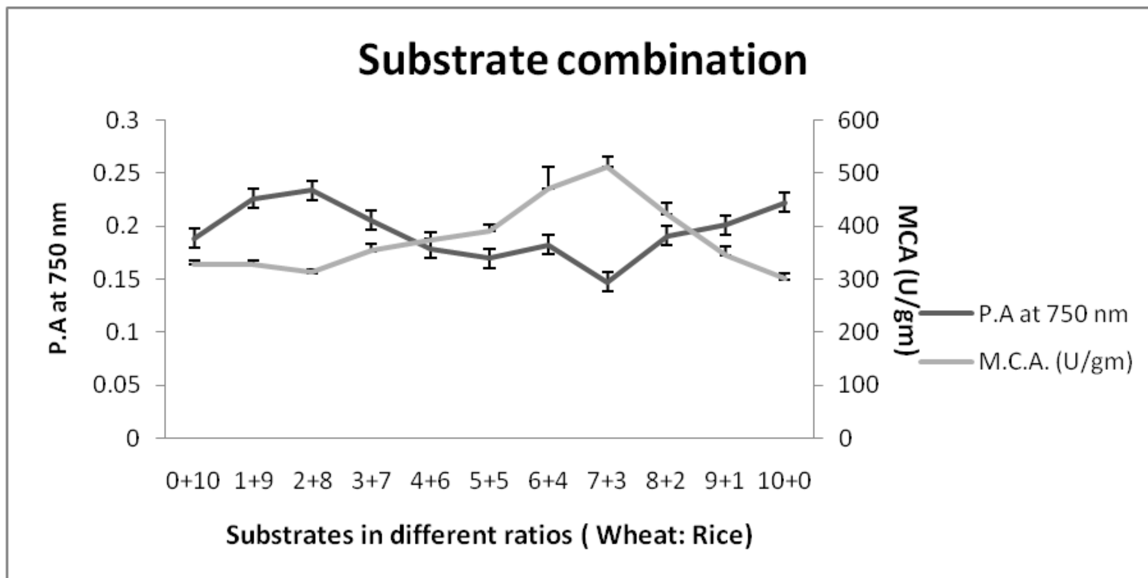

Figure 3 : Effect of substrate combination on milk clotting enzyme production under SSF. (Error bars represent standard deviations of three replicates.)

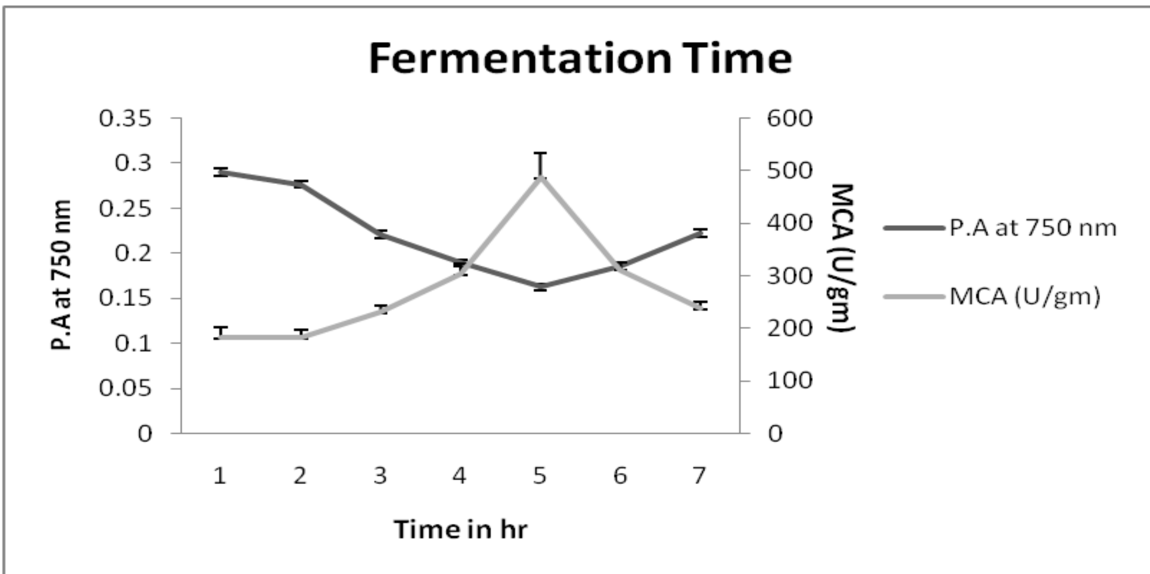

Figure 4 : Production of milk clotting enzyme under SSF at different intervals of time. (Error bars represent standard deviations of three replicates.)

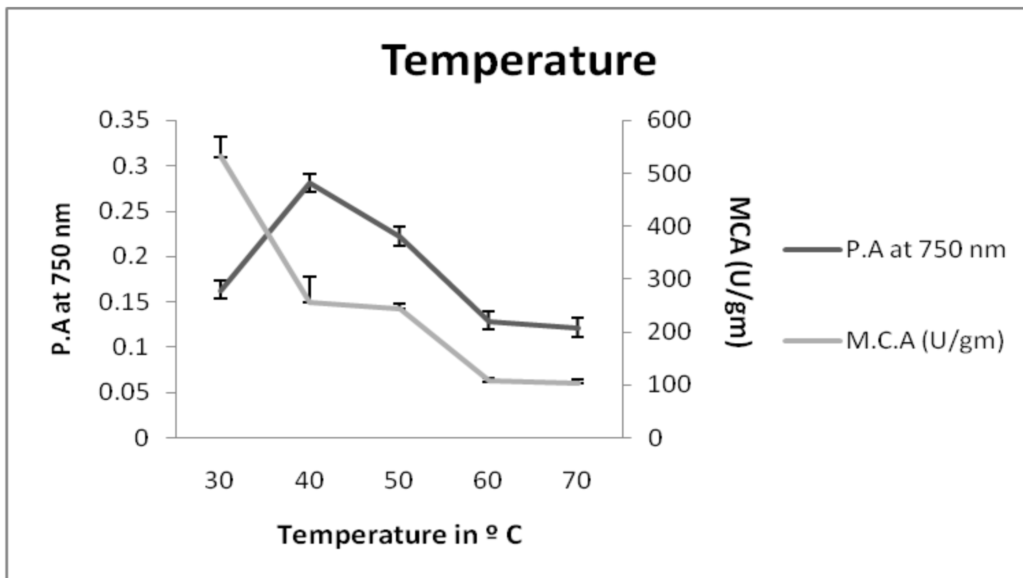

Figure 5: Effect of temperature on milk clotting enzyme production under SSF. (Error bars represent standard deviations of three replicates.)

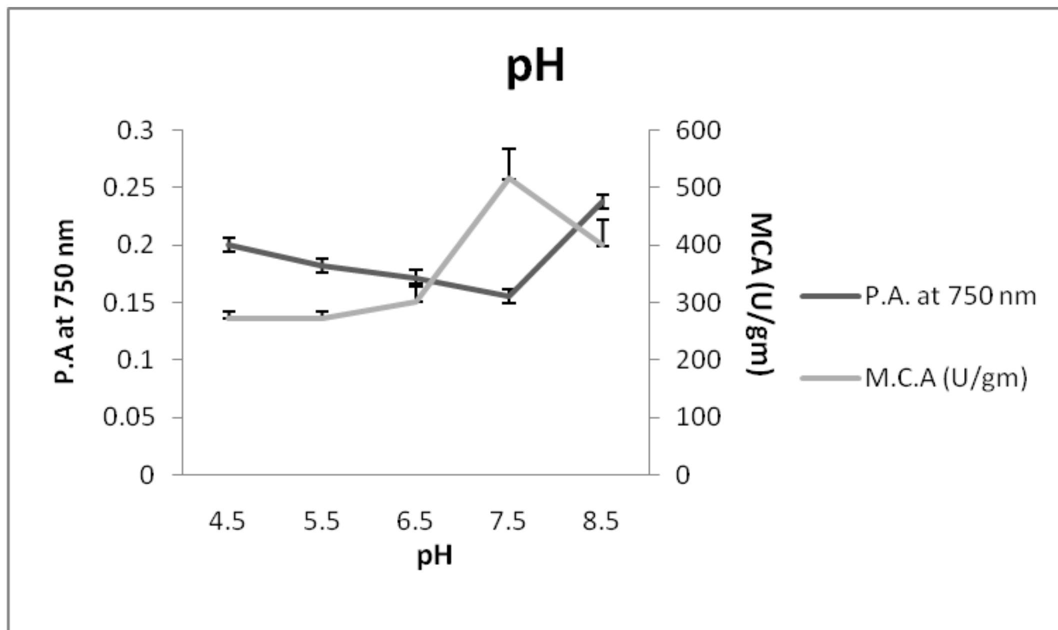

Figure 6: Effect of pH on milk clotting enzyme production under SSF
(Error bars represent standard deviations of three replicates.)

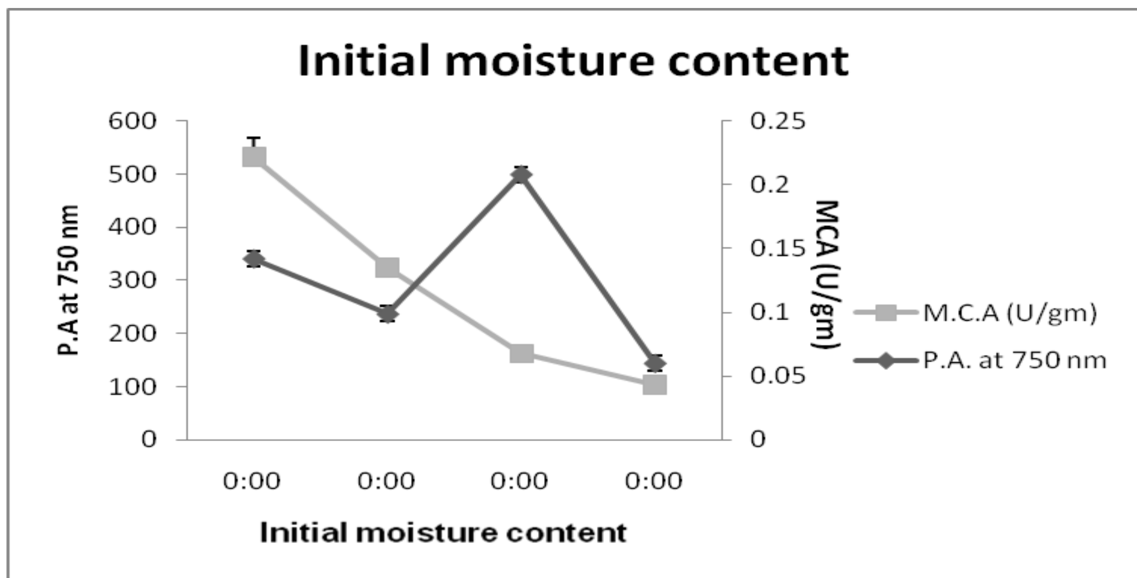

Figure 7: Effect of initial moisture content on milk clotting enzyme production under SSF.
(Error bars represent standard deviations of three replicates.)

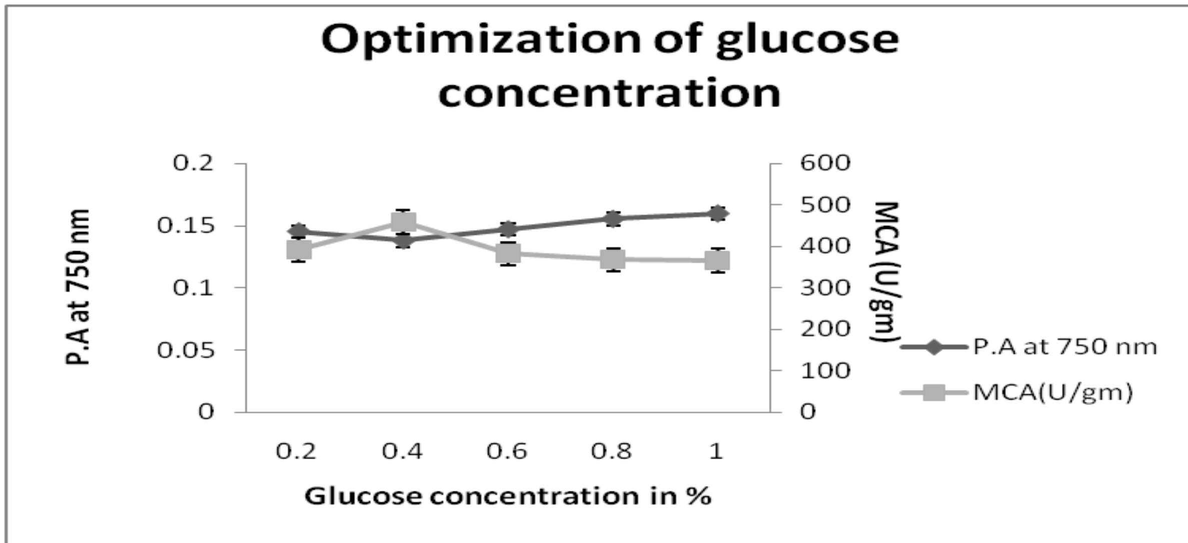

Figure 8: Effect of external Carbon sources on the production of milk clotting enzyme under SSF (Error bars represent standard deviations of three replicates.)

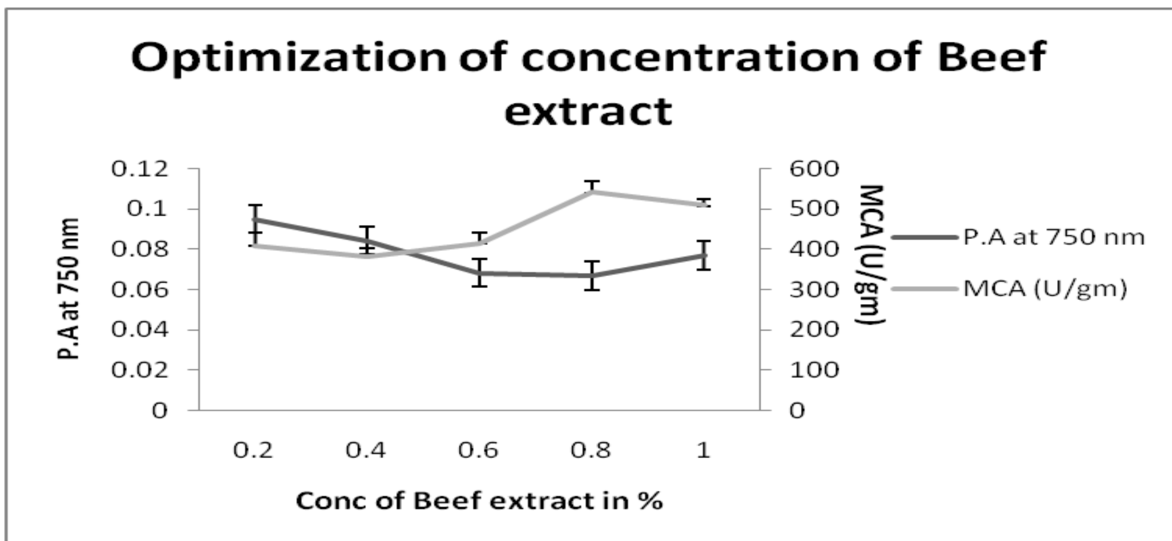

Figure 9: Effect of external Nitrogen sources on the production of milk clotting enzyme under SSF (Error bars represent standard deviations of three replicates.)

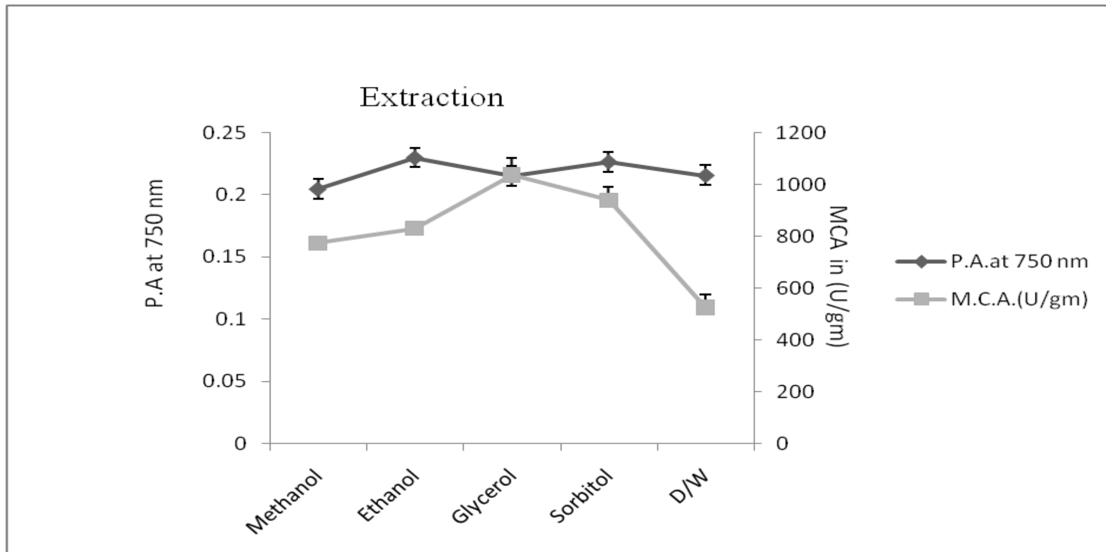

Figure 10: Effect of different organic solvents extraction of milk clotting enzyme under SSF (Error bars represent standard deviations of three replicates.)

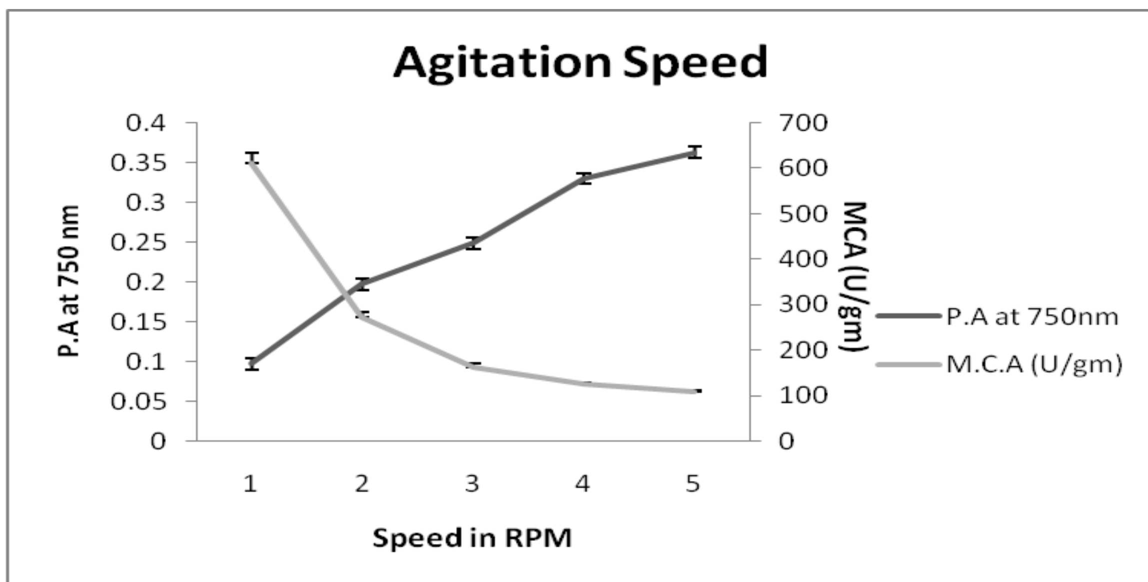

Figure 11: Effect of agitation speed on production of milk clotting enzyme under SSF. (Error bars represent standard deviations of three replicates.)

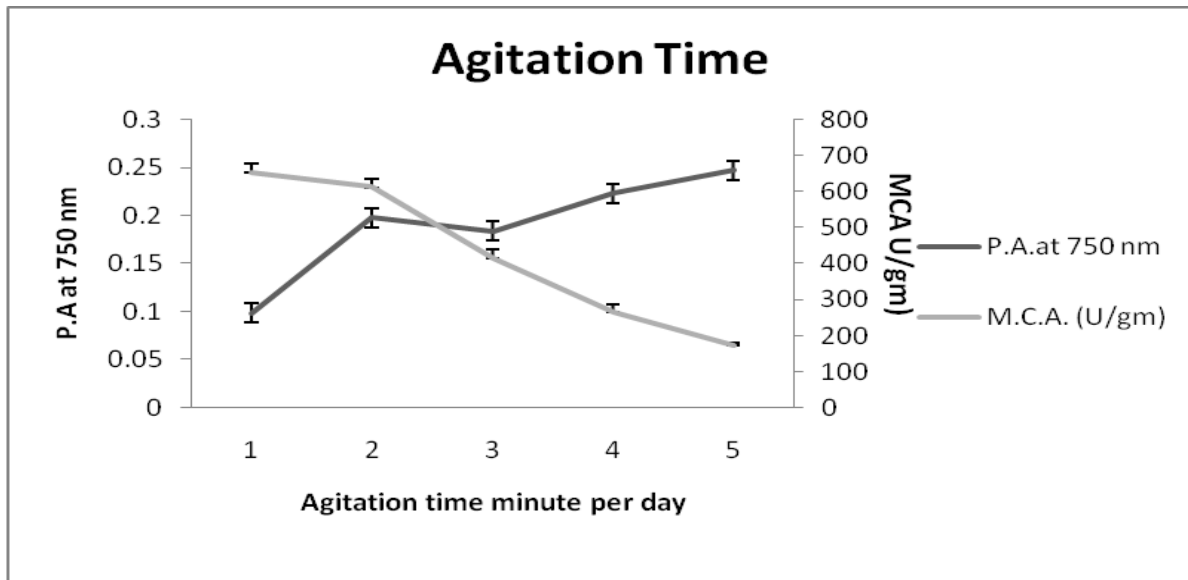

Figure 12: Effect of agitation time on production of milk clotting enzyme under SSF. (Error bars represent standard deviations of three replicates.)

VII. Tables

Table no 1:
Effect of external Carbon sources on the production of milk clotting enzyme under SSF

C sources	P.A. at 750 nm	M.C.A (U/gm)	Ratio
Maltose	0.147	394.8733 ± 9	2674
Glucose	0.134	435.1567 ± 16	3247
Sucrose	0.185	324.7633 ± 14	1755
Dextrose	0.221	351.3533 ± 16	1585
Starch	0.255	355.77 ± 20	1395

Table no 2:
Effect of external Nitrogen sources on the production of milk clotting enzyme under SSF

N Soures	P.A at 750 nm	M.C.A. (U/gm)	Ratio(MCA/PA)
Beef extract	0.077	556.3933 ± 26	7163
Pepton	0.154	435.1567 ± 16	2831
Ammonium Nitrate	0.165	130.5333 ± 5	789
Ammonium Sulphate	0.205	108.74 ± 10	531
Potassium Nitrate	0.195	117.03 ± 6	600

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