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Production of Milk Clotting Enzyme by *Penicillium camemberti*

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Abstract : In this work, the production of Milk-Clotting Enzyme was carried out using synthetic, whey and distillers sludge medium as substrates in submerged fermentation by *Penicillium camemberti*. The yield of enzyme was improved with the supplementation of lactose and casein along with the basal medium. Among the substrate, distillers yeast sludge containing casein by *Penicillium camemberti* under the static mode condition produced the highest milk-clotting activity of 0.72mcu /mg and the Proteolytic Activity of 0.59 units/ mg. The kinetics of Logistic model for cell growth and Leudeking-Piret model for product formation were evaluated on the milk clotting enzyme production by *Penicillium camemberti.*
Key words : Milk Clotting Enzyme, Aspartate Protease, Submerged Fermentation, *Penicillium camemberti*.

Introduction

The increasing global cheese production with decrease in bovine rennet production (with raised price) leads to the search for milk clotting enzymes from alternative sources[1]. Commercial preparations from the microorganisms are currently replacing the animal rennet in the production of cheese[2].Microbial rennet are produced from the microorganisms both fungi and bacteria.

Fungi have been widely used because of the enzymes that they secrete extracellularly which fecilitate the downstream processing from the fermentation medium[3]. The production of a variety of foods includes a fermentation process by using filamentous fungi. Nowadays these fermented foods are produced by selected fungal starter cultures. The most important fungal species for food fermentation are *Penicillium nalgiovense* for the production of mould fermented meat products, *P.camemberti* for the production of white cheeses and *P. roqueforti* for the production of blue veined cheeses.

The sources of raw materials for the fermentative production of milk clotting enzyme can be obtained from dairy and distillery industry wastes. Whey fermentation is particularly suitable for the production of fungal enzymes, due to their potential advantages in manufacturing products such as high yields, low environmental impact of the process, differential expression of metabolites and requirement of less expensive technology and skill[4].Distillery industry includes recyclable waste materials such as distillery yeast sludge (distiller's sludge),

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offers a better choice because of its abundance and diverse raw materials. It contains a high amount of protein (21%) and is also rich in essential amino acids[5].Because of the availability of the rich minerals , proteins and rich carbon source there is scope for using it advantageously for the various industrial byproducts[6]. Turning this solid waste into a value-added product would provide a method of disposal and an opportunity to earn a profit from the waste material.

Kinetics and modelling

Kinetic models describing the behaviour of microbiological systems can be a highly appreciated tool and can reduce tests to eliminate extreme possibilities. A meaningful way to be aware of the kinetic behaviour of the microorganisms in the fermentation process is through the kinetic parameters.

Logistic growth model

Logistic equation is a substrate independent model. The Logistic curve is sigmoidal and leads to a stationary population of size $x_s = 1/\beta$. Rate of growth of cell is proportional to the cell mass concentration present at that time. The rate will stop when the cell mass concentration reaches stationary phase. When the cell mass concentration is near the stationary phase rate will slow down.

$$x = \frac{x_o e^{kt}}{1 - \beta x_o (1 - e^{kt})} \quad \ldots (1)$$

Where x_o is the initial biomass concentration (g/l) and t is time (h). Monod and the other models predict that the growth will stop only when the limiting substrate concentration is exhausted. The advantage of this model for fermentation is that it provides the exponential phase and endogenous metabolic phase accurately

Leudeking-piret kinetic model

The kinetics of ethanol fermentation was based on the Leudeking-Piret equation originally developed for the fermentation of gluconic acid. It is an unstructured model, which combines growth and non-growth associated contribution towards product formation. This model was originally developed for the formation of lactic acid by Lactobacillus delbrucckii. The classic study of Leudeking and Piret on the lactic acid fermentation by Lactobacillus delbrucckii indicated product formation kinetics which combined growth-associated and non-growth-associated contributions:

$$r_{f_p} = \alpha_{LP} r_{f_x} + \beta_{LP} x \quad \ldots (2)$$

where r_{f_p} is the product formation rate, r_{f_x} is the biomass growth rate, α_{LP} and β_{LP} are the kinetic parameter of Leudeking-Piret model respectively.

This two parameter kinetic expression, often termed Leudeking-Piret kinetics, has proved extremely useful and versatile in fitting product formation data from much different fermentation.

$$p(t) - p_o - \beta\left(\frac{x_s}{k}\right)\left[1 - \frac{x_o}{x_s}\left(1 - e^{kt}\right)\right] = \alpha\left[x(t) - x_o\right] \quad \ldots (3)$$

This model is used for the prediction of Milk clotting Enzyme concentration during the course of fermentation. However, the above model requires biomass concentration for the prediction of product concentration[7].

The objective of this work was to evaluate and compare the production of milk clotting enzyme using synthetic basal medium, whey and Distiller's sludge as substrates by *Penicillium camemberti.*

Materials and Methods

Microorganism and its culture conditions

Penicillium camemberti(MTCC 418), a filamentous fungus was obtained from Institute of Microbial Technology, Chandigarh, India. The microorganism was grownaerobically in enrichment media containing following composition in 1000 ml distilled water: Czapek concentrate, 10 ml; K_2HPO_4, 1.0 g/l; Yeast extract, 5.0 g/l; Sucrose, 30.0 g/l. The compositions of czapek concentrate in 100ml are: $NaNO_3$, 30.0 g; KCl, 5.0 g; $MgSO_4.7H_2O$, 5.0 g; $FeSO_4.7H_2O$, 0.1 g. The pH of the medium was adjusted to 6.0 using dilute sulphuric acid. This strain was incubated at 30^{o}C for 5 days and stored at 4^{o}C.

Materials

The fresh milk whey was kindly provided by Ponlait Dairy products Ltd., Pondicherry, India. To remove the suspended particles contained in raw whey, filtration step was performed by Whatmann No. 1 filter paper. The clarified whey was used as a substrate for milk clotting enzyme production.

Fermentation experiments were also performed using distiller's sludge as substrate, obtained from EID Parry India Ltd, Nellikkuppam, Tamil Nadu, India. The substrate was sun dried, powdered and stored for further use in the experiments.

Batch Submerged Fermentation Studies

Batch submerged fermentations were carried out in 250 ml Erlenmeyer flasks with 1000 ml of production medium. The medium was distributed equally in four sterile flasks, each containing various initial concentrations of substrate. Known volume of 2 day old culture of *Penicillium camemberti* was transferred to each 100 ml of production medium in sterile conditions. The flasks were gently agitated on a shaker with a constant shaking rate at 120 rpm. All experiments were carried out in duplicate and repeated at least twice. Samples were taken from the solution at regular time intervals for the analysis of milk clotting activity, proteolytic activity and biomass concentration.

The effect of different medium components on milk clotting enzyme production was investigated using three different fermentation medium components namely plain basal medium (M1), lactose (M2) and casein (M3) along with the basal medium. The fermentation experiments were carried out with three different substrates namely synthetic medium, whey and distiller's sludge. The culture was incubated at 30^{o}C for 5 days under shaking and stationary conditions. All the experiments were carried out in duplicate and repeated at least twice.

Analysis of crude enzyme

Estimation of milk clotting activity:

Milk clotting activity was determined by the method explained by arima et al(1964)[8]using 0.1 (w/v) of rennin std. The substrate is 10g of skimmed milk powder in 0.01 mol calcium chloride. The reaction mixture contains 5 ml of skim milk and 1ml of enzyme and kept at 37^{o}C. The curd formation was observed by manually rotating the test tube from time to time. The end point is the semi liquefied film appears on the side of the test tube above the milk. The clotting time was noted.

$$MCU/mg = \frac{M}{T(\min utes)xW(g)} \quad \ldots (5)$$

Where M is the milk factor, T is the clotting time of sample (min) and Wis the grams of enzyme added to the substrate in 2.0 ml aliquot (g wt. x 2)

Estimation of proteolytic activity

Proteolytic activity was determined by the universal protease activity assay using casein as a substrate. The reaction mixture containing 5 ml of 0.65% pre incubated casein solution (37^{o}C/10min) and 1ml of enzyme (both standard and crude) was incubated for 10 min at 37^{o}C. 5 ml of TCA was added to stop the reaction and incubated at 37^{o}C for 30 min. Tyrosine standard was set up (0.2mg/ml) in the range of 0.1-0.5ml and made up to 2ml with distilled water. The test solutions were centrifuged at 4^{o}C at 10000 rpm for 10 min and the 2ml of aliquots were used for finding Proteolytic activity. To all the tubes (including standard), 5 ml of sodium carbonate, 1ml of Folin's phenol was added and incubated at 37^{o}C for 30 min and the optical density was measured at 660 nm using UV-Biospectrophotometer, which directly expresses the Proteolytic activity.[9,10]

$$Units/ml \quad enzyme = \frac{(\mu mole \quad tyro\sin e \quad equivalents \quad released)X(11)}{(1)X(10)X(2)} \quad \ldots \quad (6)$$

Where 11 is the total volume of assay(ml), 10 is the time of assay as per the unit definition (min), 1is the volume of enzyme used(ml) and 2is the volume used in colorimetric determination(ml).

Determination of protein

Protein was estimated by Lowry method(1951)[11] using BSA (200µg per ml concentration) as a standard. 0.2 to 1.0 ml of the working standards and 0.2 ml of the unknown crude sample were taken in a series of test tubes. The volume was made up to 1 ml with distilled water. 5 ml of the alkaline copper reagent was added to all the tubes and incubated for 10 min at room temperature Then 0.5 ml of Folin 's phenol reagent was added to all the tubes and incubated at dark room for 30 min and the optical density was measured for 660 nm.

Estimation of biomass concentration

Samples from the production medium were filtered through whatmann no .40 filter paper to separate the biomass. The settled biomass was collected and dried and expressing the dry weight as grams per liter of growth medium.

Results and Discussion

Effect of different medium components on the production of milk clotting enzyme by *Penicillium camemberti* using different substrates

In order to optimize the medium compositions capable of inducing high milk clotting and low proteolytic activities, three different media were tested with three different substrates. Fig 1 to Fig 3 indicates the effect of different medium components on milk clotting activity and proteolytic activity using different substrates namely synthetic medium, whey medium and distiller's sludge respectively. The plain basal medium, casein and lactose along with the basal medium were denoted as M1, M2 and M3 respectively. The results indicated that the medium containing Casein (M2) was best medium for maximum Milk clotting enzyme production using different substrates. Distiller's sludge gave maximum enzyme production than whey and synthetic medium. In all the cases, addition of Casein provided higher Milk clotting activity than the utilization of Lactose and the plain basal medium. High Milk clotting activity of 0.72 units/mg and low Proteolytic activity of 0.59 units/mg was observed in presence of Casein (M2) using distiller's sludge as substrate. Plain basal medium (M1) gave maximum Milk clotting activity (0.67 units/mg) when compared to the medium (M3) containing Lactose (0.63 units/mg). Medium containing Casein (M2) played an important role in Milk clotting enzyme production by *Penicillium camemberti* thereby it stimulates the milk clotting enzyme production and metabolism of the microorganism. The combination of casein with the substrates is an enhancer for the enzyme coagulant. As casein is the prime constituent of skim milk powder, its role in induction of enzyme synthesis is evident in these investigations. Sirmayergin[12]found that no activity was observed using Lactose as the carbon source.

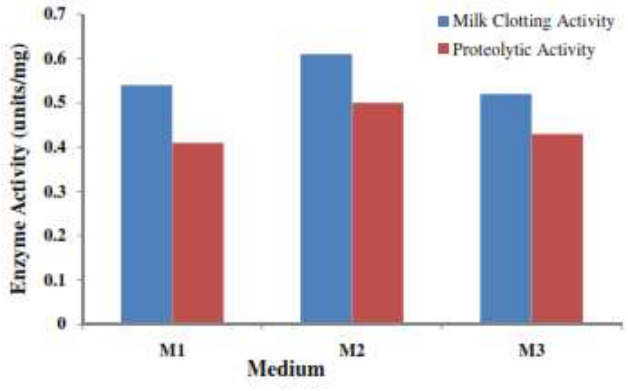

Fig 1 Effect of different medium components on Enzyme activity using Synthetic medium

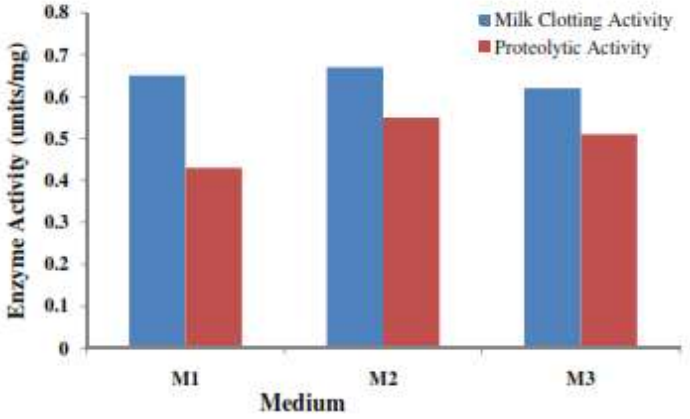

Fig2 Effect of different medium components on Enzyme activity using Whey medium

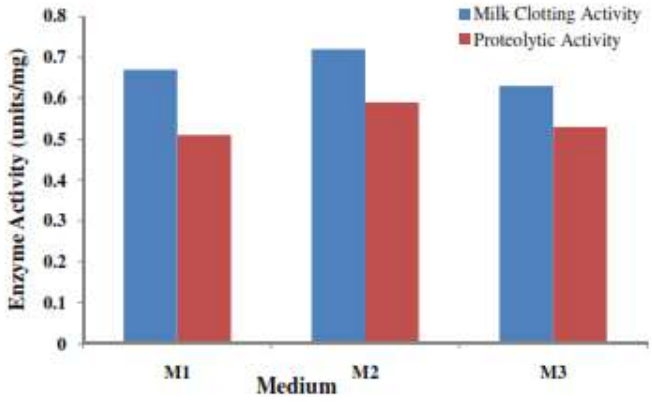

Fig3 Effect of different medium components on Enzyme activity using Distiller's sludge

Effects of Stationary and shaking condition on Milk Clotting Enzyme Production using different medium components and substrates

The intensity of agitation affects fungal morphology and noticeable influence on Milk clotting enzyme production in the bioreactor.Fig 4 and Fig 5 shows the effect of different substrate and different medium components on Milk clotting activity by *Penicillium camemberti* under stationary and shaking conditions. Fig 6 shows the time profile for the growth of *Penicillium camemberti* under shaking and stationary conditions in a medium containing casein and distiller's sludge. The maximum biomass concentration of *Penicillium camemberti* was obtained after 5 days of incubation under shaking and stationary conditions. It was found that shaking conditions suppresses the growth of *Penicillium camemberti* thereby decreasing Milk clotting enzyme production. The maximum Milk clotting activity (0.72 units/mg) was observed under stationary conditions when compared to shaking conditions (0.65 units/mg). Decreased biomass concentration and low Milk clotting activity under shaking conditions may be attributed to the effect of shear stress on fungal cells as well as the enzyme structure. It was clearly showed that the agitation conditions played an important role in Milk clotting enzyme production.

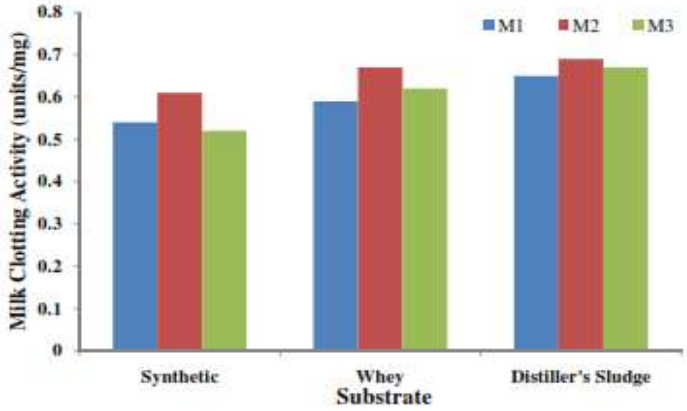

Fig 4 Effect of different substrate on Milk clotting activity by *Penicillium camemberti* under stationary conditions

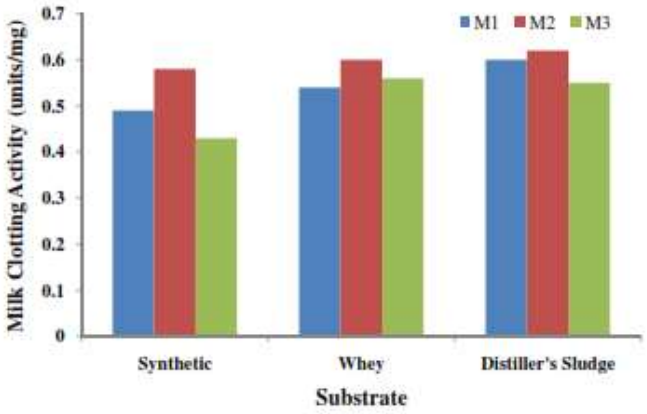

Fig 5 Effect of different substrate on Milk clotting activity by *Penicillium camemberti* under shaking conditions

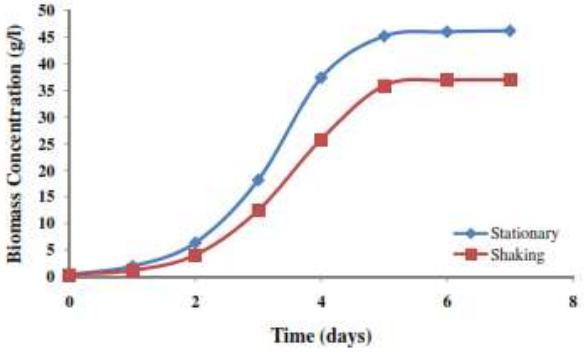

Fig 6 Growth kinetics of *Penicillium camemberti* under shaking and stationary conditions in Distiller's sludge medium

Kinetics and Modeling

Fig. 7 shows that there is a good agreement between the experimental data and the simulation results, and the Logistic model appeared to provide adequate representation of growth and fermentation kinetic

of*Penicillium camemberti*. The kinetic parameters of logistic equation constants Kc and β were found to be 0.004 h^{-1} and0.022g/l respectively. The experimental biomass concentration is well fitted with predicted biomass concentration with high regression coefficient0.9417and it is most suited for milk clotting enzyme production from distiller's by *Penicillium camemberti*.

Fig 8 shows the experimental and predicted product formation rate for milk clotting enzyme production using Leudeking-piret model. The kinetic parameter values of β and α were found to be 0.0001 and 0.026 respectively. The constants indicate that growth associated product formation depends on biomass growth and milk clotting enzyme. The experimental data fitted with predicted product formation rate with high regression coefficient of 0.9933

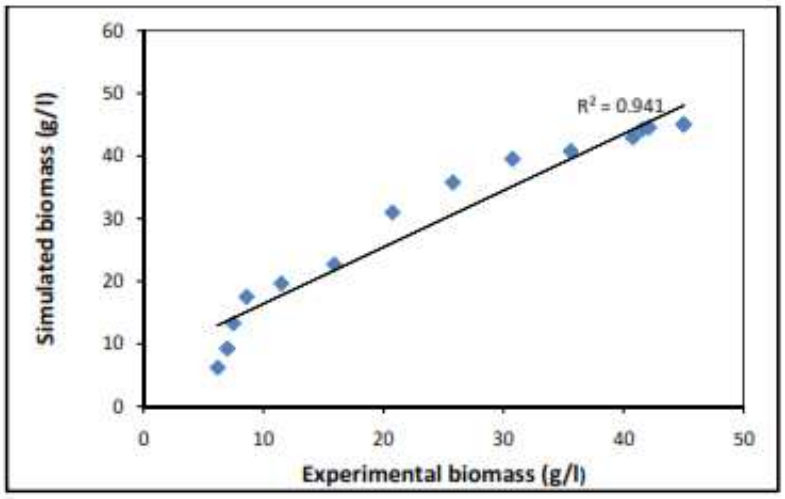

Fig 7.Comparison between experimental result and logistic growth model

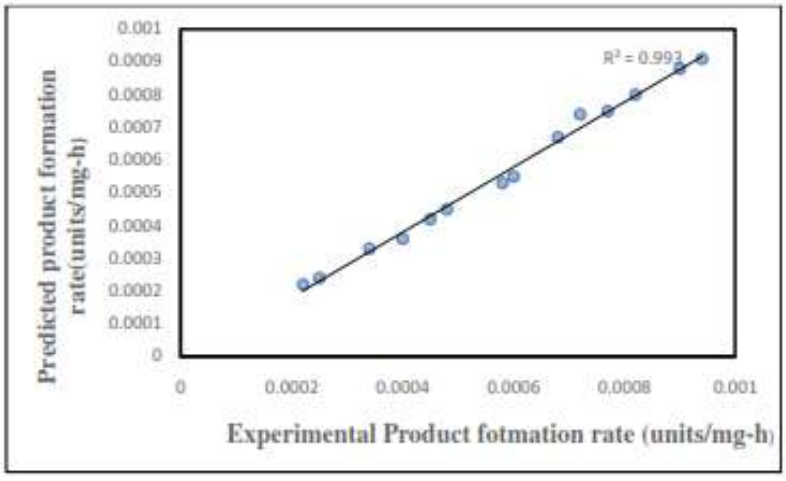

Fig 8. Experimental product formation rate and predicted product formation rate in Leudikingpiret kinetic model

Conclusions

The present study described that the comparative study of the influence of the three substrates namely synthetic medium, whey and distiller's sludge on the production of milk clotting enzyme. The Distillers sludge shows the high milk clotting activity and it is an effective substrate for the production of milk clotting enzyme by *Penicillium camemberti*. The results reported that thedistillers sludge medium containing casein understaticconditions enhanced the milk clotting activityof 0.72 unit /mg with low proteolytic activity0.59units/mg. Logistic model and Leudeking-Piret model were found to represent the experimental data

of cell growth and product formation kinetics. The results recommended that the distillers sludge is the valuable source for the production of milk clotting enzyme by the fungal culture *Penicilliumcamemebrti*.

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Author Disclosure Statement:

The authors have no conflicts of interest to declare.

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