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Research Article

MILK CLOTTING ENZYME FROM STREPTOCOCCUS LACTIS BY USING DISTILLERS SLUDGE

Santhalin Shellomith, A. S* and Preetha B

Department of Chemical Engineering, Annamalai University, Annamalai Nagar – 608 002, Chidambaram, Tamil Nadu, India

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A B S T R A C T

The growing demand for natural coagulants led to the necessity for alternate rennet substitutes, searching a new sources of proteolytic enzymes with coagulant properties. The utilization of waste as a raw material is very important for the natural balance. In this study, the distillery waste the distillers yeast sludge was used as a fermentation medium in the Production of Milk-Clotting Enzyme in submerged fermentation by Streptococcus lactis. The synthetic fermentation medium was optimized by using various carbon and nitrogen sources in both the stationary and shaking conditions then the production was carried out in distillers medium with optimized conditions under shaking at 120rpm. The maximum milk clotting activity of 0.625 units/mg and the proteolytic activity of 0.490 units/mg were obtained at 40° C. The kinetic parameters Michaelis–Menten and Lineweaver-Burk plots were evaluated in this study.

INTRODUCTION

Distillery is an important sub-unit of sugar industries. Distillers produced large amount of molasses 350,000 liter/day; yeast sludge 20,000 liter/day and spent malt grain wash 120,000 liter/day was estimated. Distillers yesast sludge posses the immense biochemical and nutitive values and it is widely used as a biofertilizer in agriculture (Thiyagarajan *et al*.,2001). Due its high nutritive value it is used as poultry feed (Sudha rameshwari and Karthikeyan 2005) and cattle feed (Sharif *et al*.,2012) because of the presence of the essential amino acids such as lysine, methionine, glycine, arginine, leucine, and histidine. The distillers yeast sludge is also used as source of single cell protein (Valentino 2015).

Milk coagulation is the important biochemical reaction in the in cheese manufacturing and milk clotting enzymes are the active coagulation agents in this process. The enzymatic mechanism involves the cleavage of kaba-casein at the peptide bond between the amino acids Phe 105-Met 106 that renders the casein micelles unstable and causes aggregation which yields a clot and formed a gel afterwards (Magda *et al*, 2017). Rennet from calf stomach is used for cheese making in olden days. It has the largest single proteolytic enzyme in food processing. Rennet not only clot the milk in the first step of cheese manufacture and also plays a vital role for the flavor and texture during cheese ripening (Zhongyang Ding 2011).

Corresponding author:* **Santhalin Shellomith, A. S
Department of Chemical Engineering, Annamalai University, Annamalai Nagar - 608 002, Chidambaram, Tamil Nadu, India

Chymosin (EC 3.4.23.4) is a milk clotting enzyme (MCE) obtained from the fourth stomach of the unweaned calf. Due to the legal problems against the animal slaughtering leads to find other alternatives to calf chymosin. In this regard, various alternatives are used for chymosin production; mainly of plants and microbial sources. (Kumar, 2005). The extracellular proteases of many microorganisms behave similarly to chymosin, and are therefore used as the potential alternatives sources for rennet. The microbial sources are better than the plant sources because most of the plant clotting enzymes give the bitter taste in cheese.Present study focused the submerged production of milk clotting enzyme in the distillers sludge medium by using *Streptococcus lactis.*

MATERIALS AND METHODS

Microorganism and its culture conditions

The bacterial culture *Streptococcus lactis (NCIM 2114)* was obtained from NCL Pune, India.. This culture was maintained by sub culturing periodically at 30°C for 24 hours and stored at 4°C.The microorganism was grown aerobically in MRS media containing following composition in 1000 ml distilled water: protease peptone, 10g; yeast extract, 5g; Beef extract, 10g; dextrose, 20g; tween 80, 1.0g; ammonium citrate, 2.0g; sodium acetate, 5.0g; Magnesium sulphate, 0.1g; Manganese sulphate, 0.05g; Dipotassium phosphate, 2.0g. The pH of the medium was adjusted to 6.5 using dilute hydrochloric acid, incubated at 30°C for 24 hours and stored at 4°C.

MATERIALS

Fermentation experiments were 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 studies.

Preparation of the rennin enzyme

The Calf chymosin (Rennin) was purchased from the Hi media for standard enzyme (800 mcu/mg). The 0.1% of standard rennin was prepared by 0.1 M solution of Calcium chloride..

Batch Submerged Fermentation Studies

Batch submerged fermentations were carried out with known volume of 1 day old culture of *Streptococcus lactis* in 100 ml of production medium in sterile conditions. The 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 carbon sources and nitrogen sources on milk clotting enzyme production was investigated using various carbon sources namely glucose (control), sucrose, maltose, lactose and starch, nitrogen sources such as yeast extract(control), casein, tryptone, urea and ammonium sulphate The fermentation experiments were carried out with two different substrates namely synthetic medium with above mentioned sources and distiller's sludge. The production was carried out at 30^{o}C for 2 days under shaking and stationary conditions. And the effect of various temperature on the production of Milk clotting Enzyme were determined.. 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) 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.

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

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, (Balls,1937 and Anson,1938).

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

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) using BSA (200µg per ml concentration) as a standard.

Determination of kinetic parameters

Michaelis-Menton plots(Dixon andWeb1979) were performed by increasing substrate concentration (Ageitos 2006).The *Vmax* and *Km* values of the enzymatic samples were obtained using using the Lineweaver -Burk method (Dixon and Web 1979).

Estimation of biomass concentration

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

RESULTS AND DICUSSION

Production of Milk clotting enzyme was optimized by one time approach with one variable to determine the factors affecting the enzyme production under the suitable growth conditions (30°C, pH6.5). Various Carbon and nitrogen sources were supplemented with fermentation medium to find out the suitable Carbon and nitrogen sources for the Enzyme production

Effect of Carbon sources on the production of milk clotting activity by Streptococcus lactis

To determine the effects of carbon sources on the production of Milk clotting enzyme by *Streptococcus lactis*, the batch fermentation was carried out in only the basal medium which contains glucose and the other carbon sources namely sucrose, maltose, lactose and starch were supplemented in the production medium. Fig1 shows the glucose and sucrose produce higher MCA than the other sources such as lactose, maltose and starch. Carbon sources enhances the enzyme production based on the metabolism of the microorganism. The maximum milk clotting activity was observed in glucose containing medium because the monosaccharide's are easily up taken by the metabolic processes in all the living organism. The results are accordance with other reports such as glucose influence the enzyme production in *M.meihei* (Silveria *et al*. 2005) and 9 fold increase in MCA with glucose by *Bacillus subtilis* was observed by Dutt *et al* (2008). Therefore glucose with basal medium appears to be the favourable carbon source for the production of milk clotting enzyme by *Streptococcus lactis.*

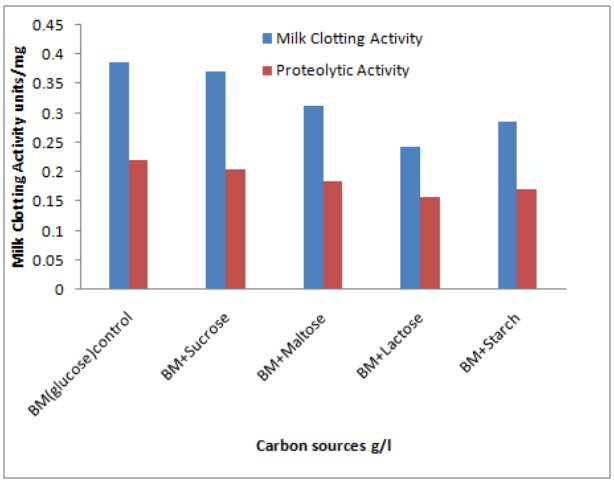

Fig 1 Effect of Carbon sources on the production of milk clotting activity by Streptococcus lactis

Effect of Nitrogen sources on the production of milk clotting activity by Streptococcus lactis

Nitrogen sources are the important factor for the growth of the microorganisms which influence the production of enzyme. In this study, the yeast extract, casein and tryptone are used as organic source, urea and ammonium sulphate as inorganic source for nitrogen sources with basal medium. Fig 2 shows the Yeast extract with peptone and casein produce higher MCA than the tryptone, urea and ammonium sulphate contained medium. The high milk clotting activity was observed in the casein containing medium. The results already reported that the highest MCA was acheived by casein and followed by yeast extract in the fermentation with *Rhizomucor nainitalensis* (Khademi *et al*.,2013). Dutt *et al* (2008) reported that the increased Milk clotting activity was found in the casein, peptone and yeast extract when compared to inorganic nitrogen sources such as urea and ammonium sulphate by fermentation with *B.subtilis*.

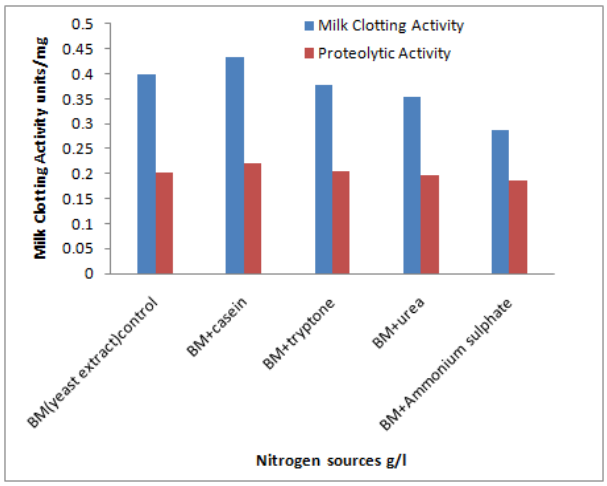

Fig 2 Effect of Nitrogen sources on the production of milk clotting activity by Streptococcus lactis

Effect of stationary and shaking conditions on the production of milk clotting activity by Streptococcus lactis

The submerged fermentation was carried out with the optimized carbon and nitrogen sources namely glucose and casein with basal medium under shaking at 120rpm and stationary conditions. Fig 3 shows the maximum biomass production in the shaking conditions (9.3g/l) than the stationary conditions (8.2g/l). The increased biomass at the shaking condition indicates the metabolic rate of the microbial population influenced by the aeration thereby increasing the biomass concentration. Agitation is effective for the leaching process to reduce enzyme adhesion to cell biomass and also the substrates was immensely mixed with fermentation. (Ahamed 2008)

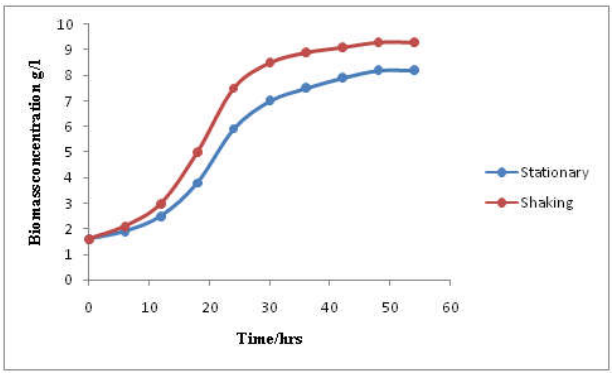

Fig 3 Effect of stationary and shaking conditions on the Biomass concentration by *Streptococcus lactis* in optimized basal medium

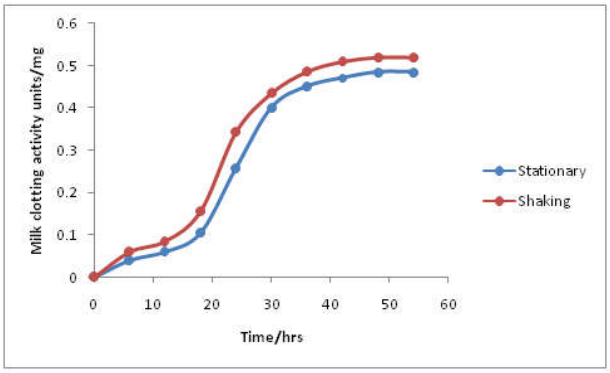

Fig 4 Effect of stationary and shaking conditions on the Milk clotting activity by *Streptococcus lactis* in optimized basal medium

Fig 4 shows the higher milk clotting activity (0.520units/mg) under shaking condition than the stationary (0.485units/mg) fermentation. The increased biomass influences the increased milk clotting activity due to more production of milk cloting enzyme.

Effect of temperature on the production of Milk clotting Enzyme

The production was carried out in various temperature 20,25,30,35,40,45,50,55 and 60° with optimized medium sources under shaking mode at 120 rpm. The maximum milk clotting activity of 0.599 units/mg was obtained at 40 C°. There is a loss of activity towards increasing temperature range indicates the inhibitory effect of higher temperature. It is well accordance with the results repported by Ahamed (2008) by bacterial *Bacillus megaterium* which produced high milk clotting enzyme at 40 °C.

Submerged production in Distillers sludge

Submerged Production in Distillers sludge was carried out under shaking condition in distillers sludge with basal medium consists of optimized carbon and nitrogen sources at 120 rpm with 40°C. The Milk clotting activity (MCA), Proteolytic activity (PA), MCA/PA ratio, Protein and Biomass were obtained 0.625 units/mg, 0.490 units//mg, 1.27, 0.702 and 29.2g/l were obtained respectively. High milk clotting activity was observed in the distillers sludge medium when compared to the synthetic basal medium. Fig 5 shows the milk clotting

activity and proteolytic activity of the distillers sludge fermentation. The milk clotting activity (0.625units/mg) is higher than the proteolytic activity (0.490units/mg) which indicates the potent source of rennet for cheese making.

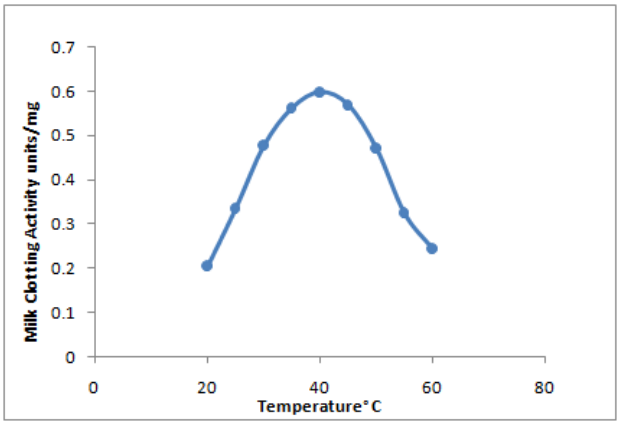

Fig 5 Effect of Temperature on Milk clotting activity by Streptococcus lactis in distillers sludge

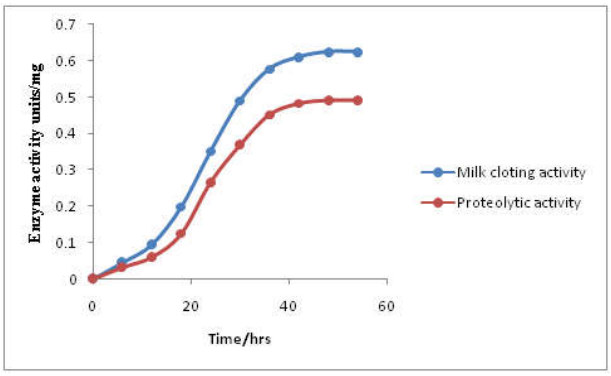

Fig 6 Milk clotting activity and Proteolytic activity by Streptococcus lactis in distillers sludge

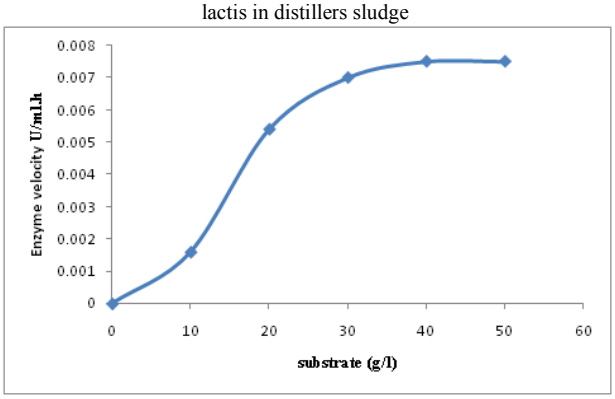

Fig 7 Michaelis–Menten plot

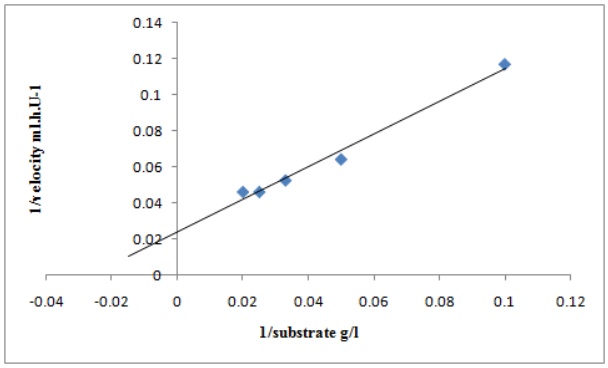

Fig 8 Lineweaver-Burk plot

Km and Vmax determination

Fig 7 and 8 shows Michaelis-Mentons plot and the Line weaver bulk plot which indicates the activity of the enzymatic sample was measured in the presence of increasing concentrations of substrate in the typical hyperbolic velocity saturation curve with the *Km* value of 39.62 and the *Vmax* value of 0.023.

CONCLUSIONS

The supplementation of glucose and casein with Distillers sludge at 40°C influences the production of milk clotting enzyme. The utilization of distillers sludge into the valuable bio products is the best way to overcome the distillery discharges into the environment. Due its high nutritive value, it is an effective substrate for the production of milk clotting enzyme. The results reported that the distillers sludge medium under shaking conditions enhanced the milk clotting activity of 0.625 units//mg with low proteolytic activity 0.490 units/mg. The *streptococcus lactis* is the suitable microorganism for this enzyme production at 40° C.

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Author Disclosure Statement

The authors have no conflicts of interest to declare.

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