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Research Article

PRODUCTION OF MILK CLOTTING ENZYME BY *STREPTOCOCCUS LACTIS* UNDER SUBMERGED FERMENTATION

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ABSTRACT

The present study deals the production of Milk-Clotting Enzyme using synthetic, whey and distillers sludge medium as substrates in submerged fermentation by *Streptococcus lactis* the production of enzyme was improved with the addition of lactose and casein along with the basal medium. Distillers yeast sludge containing casein by *Streptococcus lactis* under the shaking condition produced the highest Milk clotting activity of 0.608 units/mg and the Proteolytic Activity of 0.488 units/ mg. The high milk clotting activity with low proteolytic activity is the best condition for rennet strength in cheese making. The kinetics of Logistic model for cell growth and Leudeking-Piret model for product formation were evaluated on the milk clotting enzyme production by *Streptococcus lactis*.

INTRODUCTION

Milk clotting enzymes play an important role in cheese making Industries. Historically it means of preserving milk. Group of microbes transform into the milk into cheese and they are active participants in the development of cheese throughout the aging process. Milk clotting enzymes are biochemically known as Aspartic proteinases. Aspartic proteinases are an important class of proteinases which are widely used as milk-coagulating agents in industrial cheese production. They are available from a wide range of sources including mammals, plants, and microorganisms. Aspartic proteinases (EC 3.4.23), which are called as acid proteinases or aspartyl proteinases include two aspartic acid residues within their active sites. These two aspartic acid residues play an active role for their catalytic activity (SirmaYegin *et al.* 2011) during the mechanism of enzyme substrate reactions.

The term 'rennet' was originally used to describe the milk-clotting enzyme preparation from calf stomach, which contains the active digestive enzyme called chymosin (rennin). At present, the term 'rennet' is used broadly to describe milk-clotting enzymes.(Farkye,2003) Enzymes extracted from the fourth stomach (abomasum) of suckling calves (rennet) have traditionally been used as milk coagulants for cheese production.

A world shortage of bovine rennet, due to the increased demand for cheese, leads to the search for alternative milk coagulants. The global market for the production of microbial enzymes for use in dairy industries are considerably large, but there is a limited number of enzyme producers to develop the dairy industries. Microbial rennet are produced from the microorganisms both fungi and bacteria. Commercial preparations from the microorganisms are currently replacing the animal rennet in the production of cheese (Delima *et al*, 2008)

The sources of raw materials for the fermentative production of milk clotting enzyme can be obtained from dairy and distillery industry wastes. Distillery sludge contains a high concentration of essential major plant nutrients nitrogen,. phosphorus, potassium and micro nutrient such as trace elements. (Suthar,2008). Dried yeast sludge is a promising source of protein and water soluble carbohydrate besides other nutrients and also contains B-complex vitamins (Arora, 1999). The nutritive value of dried yeast sludge is the potential source for the production of value-added product would provide a method

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of disposal and an opportunity to earn a profit from the waste material.

Many works carry out on the whey offered new and diverse applications for the extraction of microbial proteases from bacteria, fungi and yeast using processes like solid-state and submerged fermentation (Potumarthi *et al*., 2007). Whey is a complex protein derived from milk, as a functional food with a number of health benefits. The biological components of whey, including lactoferrin, betalactoglobulin, alpha-lactalbumin, glycomacropeptide, and immunoglobulins (Marshall 2004). Whey contains a large quantity of water soluble carbohydrates such as lactose and has already been used in various research works as a carbon source (Assenat and Luquet, 1985; Mechakra *et al*., 1999). Whey fermentation is suitable for the production of enzymes, due to their potential effects in manufacturing products with high yields and low environmental impact of the process (Abeer *et al* 2015)

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 (Bailey and Ollis,1986)

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 *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

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.

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 prepared was by diluted in 0.1 M solution of Calcium chloride and used for the Milk coagulation.

Batch Submerged Fermentation Studies

Batch submerged fermentations were carried out with 100 ml of production medium in 250 ml Erlenmeyer flasks. Known volume of 1 day old culture of *Streptococcus lactis* 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°C for 2 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) 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°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.

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

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°C/10min) and 1ml of enzyme (both standard and crude) was incubated for 10 min at 37°C. 5 ml of TCA was added to stop the reaction and incubated at 37°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°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°C for 30 min and the optical density was measured at 660 nm using UV-Biospectrophotometer, which directly expresses the Proteolytic activity (Balls,1937 and Anson,1938).

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

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. 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 Streptococcus lactis using different substrates

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 6 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.

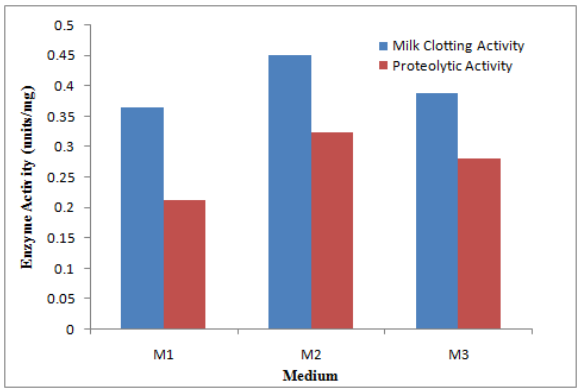


Fig 1 Effect of different medium components on Enzyme activity using Synthetic medium in Stationary condition

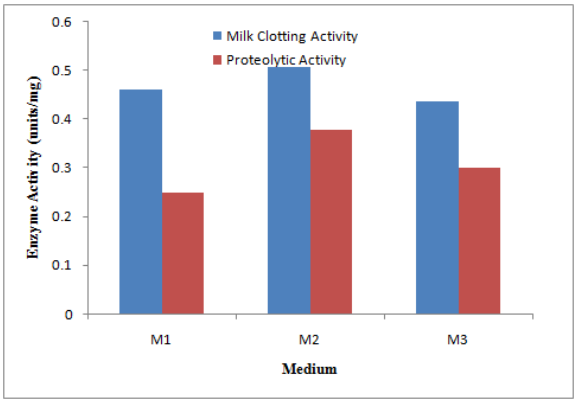


Fig 2 Effect of different medium components on Enzyme activity using Synthetic medium in Shaking condition

The plain basal medium, casein and lactose along with the basal medium were denoted as M1, M2 and M3 respectively. It was found that the medium containing Casein (M2) shows the

best medium for maximum Milk clotting enzyme production by different substrates.

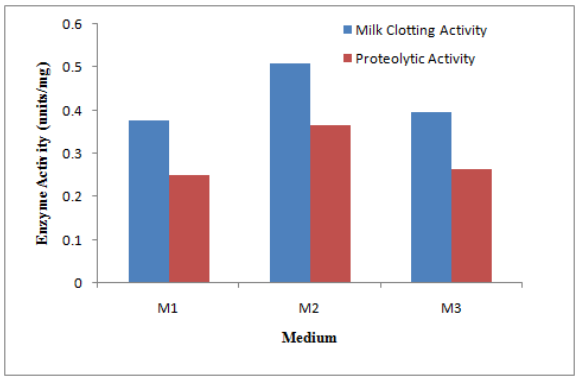


Fig 3 Effect of different medium components on Enzyme activity using Whey medium in Stationary condition

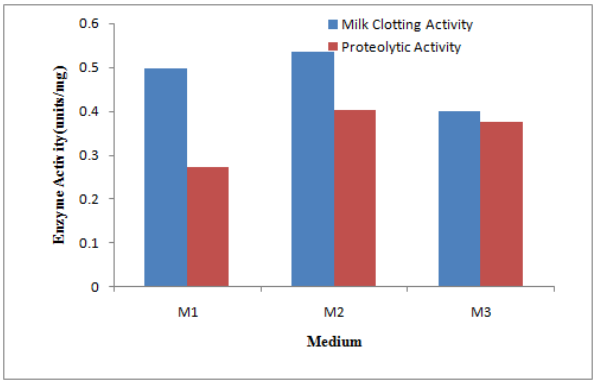


Fig 4 Effect of different medium components on Enzyme activity using Whey medium in Shaking condition

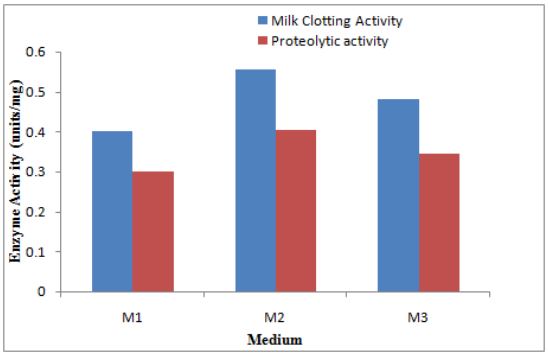


Fig 5 Effect of different medium components on Enzyme activity using Distiller's sludge in Stationary condition

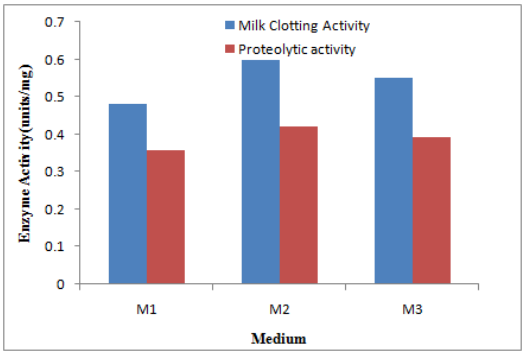


Fig 6 Effect of different medium components on Enzyme activity using Distiller's sludge in Shaking condition

It was found that the Distiller's sludge gave maximum enzyme production than whey and synthetic medium. Addition of Casein provided higher Milk clotting activity than the utilization of Lactose and the plain basal medium. High Milk clotting activity of 0.60units/mg and low Proteolytic activity of 0.412 units/mg was observed in presence of Casein (M2) using distiller's sludge as substrate when compared to the plain basal medium (M1-0.508units/mg) and lactose medium (M3-0.525units/mg) .Medium containing Casein (M2) played an important role in Milk clotting enzyme production . The combination of casein with the substrates act as a enzyme coagulant for the clotting mechanism. The role of casein in enzyme synthesis is evident in these investigations.

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

The intensity of agitation influences the Milk clotting enzyme production .Fig 1 to Fig 6 shows the effect of different substrate and different medium components on Milk clotting activity by *Streptococcus lactis* under stationary and shaking conditions. The maximum Biomass concentration 25.3g/l was obtained in the M2 medium when compared to M1(20.4g/l) and M3 (17.5g/l) under shaking conditions. The maximum Enzyme production was obtained by *Streptococcus lactis* under shaking conditions in a medium containing casein and distiller's sludge. It was found that shaking conditions influences the growth of *Streptococcus lactis* thereby increasing Milk clotting enzyme production. The maximum Milk clotting activity (0.60 units/mg) was observed under shaking conditions when compared to stationary conditions (0.554 units/mg). Fig 7 and 8 shows the biomass concentration and milk clotting activity with respect to time in shaking and stationary conditions.

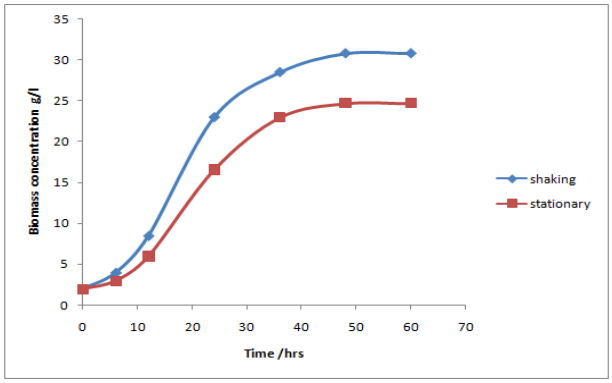


Fig 7 Effect of static and shaking cultures on Biomass concentration by *Streptococcus lactis*

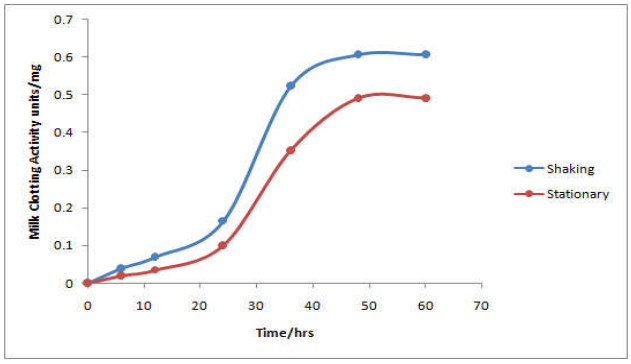


Fig 8 Effect of static and shaking cultures on Milk clotting activity by *Streptococcus lactis*

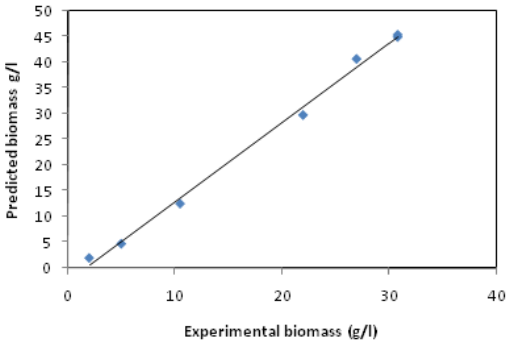


Fig 9 Comparison between experimental result and logistic growth model

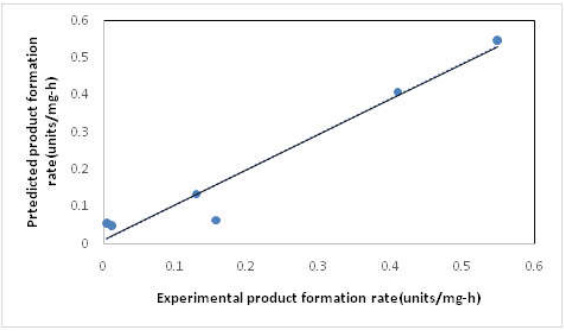
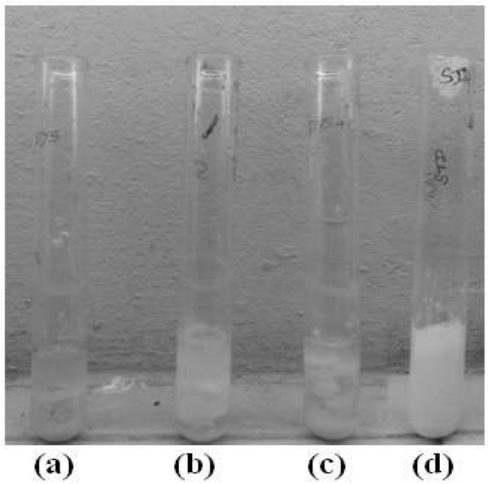


Fig 10 Experimental product formation rate and predicted product formation rate in Leudikingpiret kinetic model

Decreased biomass concentration and low Milk clotting activity under stationary conditions showed that the agitation conditions played an important role in Milk clotting enzyme production. Fig 11 shows the milk clotting observation of the synthetic, whey and distillers yeast sludge along with standard rennin (calf chymosin). The standard rennin gives the immediate coagulation within 2 min with fine texture and settled within 4min. Coagulation was obtained after 5 minutes in synthetic medium. Complete milk clotting was observed within 4 minutes in distillers sludge medium with good texture and settled within 6 minutes. In the case of whey, precipitation appeared after 8 minutes with loose texture.



(a)synthetic
(b)whey
(c) distillers sludge
(d) standard rennin

Fig11 Milk clotting observation for synthetic, whey, distillers sludge medium and standard rennin (calf chymosin)

The confirmatory experiments were carried out under optimized medium components in the Bioreactor (APPLIKON Biotech, Holland- 2L Capacity) at 120rpm with casein containing distillers sludge medium. Milk Clotting Activity, Proteolytic Activity, the ratio of MCA/PA, Biomass and protein content were found to be 0.608units/mg,0.488 units/mg,1.245,30.8g/l and 0.972mg/ml. The MCA of the confirmatory experiments well accordance with MCA of (0.60units/mg) one variable at a time and keeping the others constant.

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 *Streptococcus lactis*. The kinetic parameters of logistic equation constants Kc and β were found to be 0.150 h^{-1} and0.020g/l respectively. The experimental biomass concentration is well fitted with predicted biomass concentration with high regression coefficient 0.996 and it is most suited for milk clotting enzyme production from distiller's by *Streptococcus lactis*.

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 0.0002 and 0.017 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 0.9901

CONCLUSIONS

The Distillers sludge shows the high milk clotting activity than the synthetic and whey medium. It was found that the distillers sludge is an effective substrate for the production of milk clotting enzyme by *Streptococcus lactis*. The results reported that the distillers sludge medium containing casein under shaking conditions enhanced the milk clotting activity of 0.608 unit /mg with low proteolytic activity 0.488units/mg. Logistic model and Leudeking-Piret model were found to represent the experimental data of cell growth and product formation kinetics. The results suggested that the distillers sludge is the valuable source for the production of milk clotting enzyme by the bacterial culture *Streptococcus lactis.*

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Author Disclosure Statement

The authors have no conflicts of interest to declare.

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