PHARMACIA
Vol.-02, Issue -2 Year Dec. 2014
ISSN. 0976-9692

OPTIMIZATION OF INCUBATION TIME, R.P.M, INDUCER, INHIBITOR & IMMOBILIZATION ACTIVITY FOR β-GALACTOSIDASE PRODUCTION USING ISOLATED AND MUTATED STRAINS FROM DAIRY WASTE THROUGH FERMENTATION

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ABSTRACT

This paper investigates the production & optimization of β-galactosidase enzyme using synthetic medium by isolated wild strains (S1, S2) & mutated strains (M1, M2) through SSF & SmF. Among the different cell disintegration methods used, the highest specific activity was obtained when the cells were permeabilized using isoamyl alcohol. Wet lab experiments were performed to investigate the effects of incubation time on Vogel's medium for β-galactosidase enzyme activity using S1, S2 & M1, M2 strains through SSF. SmF experiments were performed for effects of incubation time in YLK2Mg medium on β-galactosidase enzyme activity using S1, S2 & M1, M2 strains. Effect of R.P.M speed, inducer concentration, inhibitor concentration on β-galactosidase enzyme production was also done using S1, S2 & M1, M2 strains. Results were found to be very appreciable and discussed in the paper.

***Keywords*:** *β-galactosidase, cell disintegration, permeabilized, SSF, SmF.*

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INTRODUCTION:

The importance of lactose of milk in imparting characteristic flavor and texture to milk and milk products, as well as its contribution towards their nutritive value, are well recognized *(Mozumder et al, 2011)*. On the other hand, the problems associated with whey disposal, lactose crystallization in frozen concentrated desserts, and milk consumption by lactose-intolerant populations of the world has drawn the attention of several research workers (*Rao & Dutta, 1977*). This has led to the survey of many microorganisms (*Acevedo et al, 1996*) with a view to selecting microorganisms with high potentials for producing β-galactosidase, the enzyme that initiates the breakdown of the sugar lactose. Lactose is a type of sugar found in milk. The ability of β-galactosidase to hydrolyze the β, 1-4-D-galactosidic linkage is one of the most promising applications of enzymes in food processing. The enzyme hydrolyses lactose in milk to glucose and galactose, thereby increasing its digestibility and sweetness (*File://A:SI/Beta galactosidase.htm, 2004*). The enzyme β-galactosidase has gained significant importance in the last decades because of its ability to produce hydrolysed lactose and whey products for the following reasons:

1. Interest in immobilized derivatives of β-galactosidase stems mainly from their application to hydrolyze lactose from whey, leading to the use of resulting sugars. This process is important from nutritional and technologic point of view and it can also solve the pollution problem caused by whey disposal (*Ovsejevi & Brena, 1995*).
2. Low solubility, tendency to crystallize, and low sweetness make lactose a less attractive food sugar. Enzymatically hydrolyzed lactose offers a better alternative for commercial use in food products.
3. β-galactosidase is produced by various microorganisms. Many fungal and yeast species have been traditionally used for the production of the enzyme, particularly *A.niger* and *K.fragilis*. The β-galactosidase enzymes produced by *Kluyveromyces sp.* are intracellular (*Singh, 1998*).

Why β-galactosidase?

The problems associated with whey disposal, lactose crystallization in frozen concentrated desserts, and milk consumption by lactose-intolerant populations of the world has drawn

the attention of several research workers to work in this area (*Rao & Dutta, 1977*).

Sources of β-galactosidase:

β- galactosidase is widely distributed in nature and is produced by Animals, Plants and Micro-organisms:

- Various genera of microorganism can produce β-galactosidase but the filamentous fungi (*Aspergillus niger*) & yeast (*kluyveromyces fragilis*) being used mostly.
- In plants β-galactosidase occurs in the emulsions of some Rosaceae (almond, Peach, apricot and apple tree), kefir grains, tips of wild roses and seeds of alfalfa and coffee.
- In animals it is found in the intestine of dog, rabbit, calf, sheep and goat and in snail.

MATERIALS & METHODS

Isolation of wild strains (yeast) from dairy waste samples:

Isolation of β-galactosidase producing strains was performed by the routine microbiological procedure and inoculation on a solid medium. Quantitative analysis for the presence or absence of specific microorganisms was done by plating on selective media. The isolated wild strains (S1 & S2) from dairy waste were sub cultured once a week and stored in a refrigerator at 4^{o}C.

Maintenance of Isolated strains:

Previously isolated β-galactosidase producing strains S1, S2 and mutated strains M1 & M2 were streaked on to YEPL agar slants contains; Yeast extract, peptone and lactose. The slants were incubated at 30˚ C overnight and then stored at 4˚C for further use.

Preparation of inoculums:

The isolated strains were initially grown in YEPL medium for inoculum development. 3% of the medium were used as inoculums for the inoculation of the fermentative medium.

Production of β -galactosidase under SmF:

YLK_2Mg medium was used for the determination of enzymatic activity of β–galactosidase under SmF. 1L of YLK_2Mg medium contains; Yeast extract–1gm, Lactose–10 gm, Peptone–1.5 gm, K_2HPO_4 -1 gm, $MgSO_4.7H_2O$–1.0 gm.

Production of β -galactosidase under SSF:

Vogel's medium was used for the determination of enzymatic activity of β–galactosidase under SSF. 1 L Vogel's medium contains; KH_2PO_4–0.5gm, NH_4NO_3–0.2gm, $(NH_4)_2SO_4$–0.4gm, $MgSO_4.7H_2O$–0.02gm, Peptone–0.1gm, Yeast Extract – 0.2gm, Glucose-15gm.

Assay of enzyme activity:

The β-galactosidase activity was measured spectrophotometrically by using ortho-nitrophenyl-β-D-galactopyranosidase. A sample of .05 mL was mixed with 2.0 mL of 8.3mM ONPG in sodium acetate buffer of pH 6.5, & incubated at 15 min for 37ºC. The reaction was stopped by adding 1 mL of 10% sodium carbonate solution, then mixed by swirling, diluted to 10 mL with distilled water, & mixed again. Librated o-nitrophenol was measured spectrophotometrically at 420 nm. The absorbance was read at 420 nm and the amount of ONP is to be calculated using standard curve. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 μm of ONP per min at the temperature of 55°C (*Paula et al 2008*).

Standard ONP stock:

0.1% O-nitrophenol was used as standard ONP stock solution. Different volumes of ONP ranging 0.2-2.0ml were pipette out in separated test tubes and makeup the volume of 4 ml in each test tube with distilled water. All the tubes were incubated for 15 minutes at room temperature. The optical density was measured at 420nm against water blank. The plot between concentrations versus optical density was used as standard curve.

Immobilization of β -Galactosidase

Enzymes are catalytic molecules they are not directly used up by the process in which they are used. However due to denaturation, they do loose activity with time. Therefore they should be stabilized against denaturation. When the enzymes are used in a soluble form they contaminate the product, and its removal may involve extra purification costs. In order to eliminate wastage and improve productivity, the enzyme and product can be separated during the reaction. The enzyme can be imprisoned allowing it to be reused but also preventing contamination of the product. Unstable enzymes may be immobilized by being attached to or located within an insoluble support, therefore the enzyme is not free in solution. Once attached, an enzyme's stability is increased, possibly its ability to change shape *(Tischer, 1999)*.

One of the best carriers to immobilize enzyme is chitosan, a linear polymer of β-(1, 4)-2-amino-2-deoxy-D-glucopyranose derived from chitin by deacetylation. Chitosan has many useful features, such as hydrophilicity, biocompatibility, and biodegradability. In recent years, chitosan was generally used to immobilize various enzymes including β-galactosidase due to the presence of reactive amino functional groups generally with glutaraldehyde as

crosslinking reagent *(Patel et al, 2004)*. In this the activated chitosan was firstly obtained with 2, 4, 6 trichloro1, 3, 5-triazine as crosslinking reagents in non aqueous medium and its application to immobilize β-galactosidase was also investigated. Under the optimum conditions, the yield of the enzyme activity was determined and the results were compared with that from the traditional coupling reagent-glutaraldehyde *(Balakumaran et al, 2012)*.

Chitin and Chitosan preparation

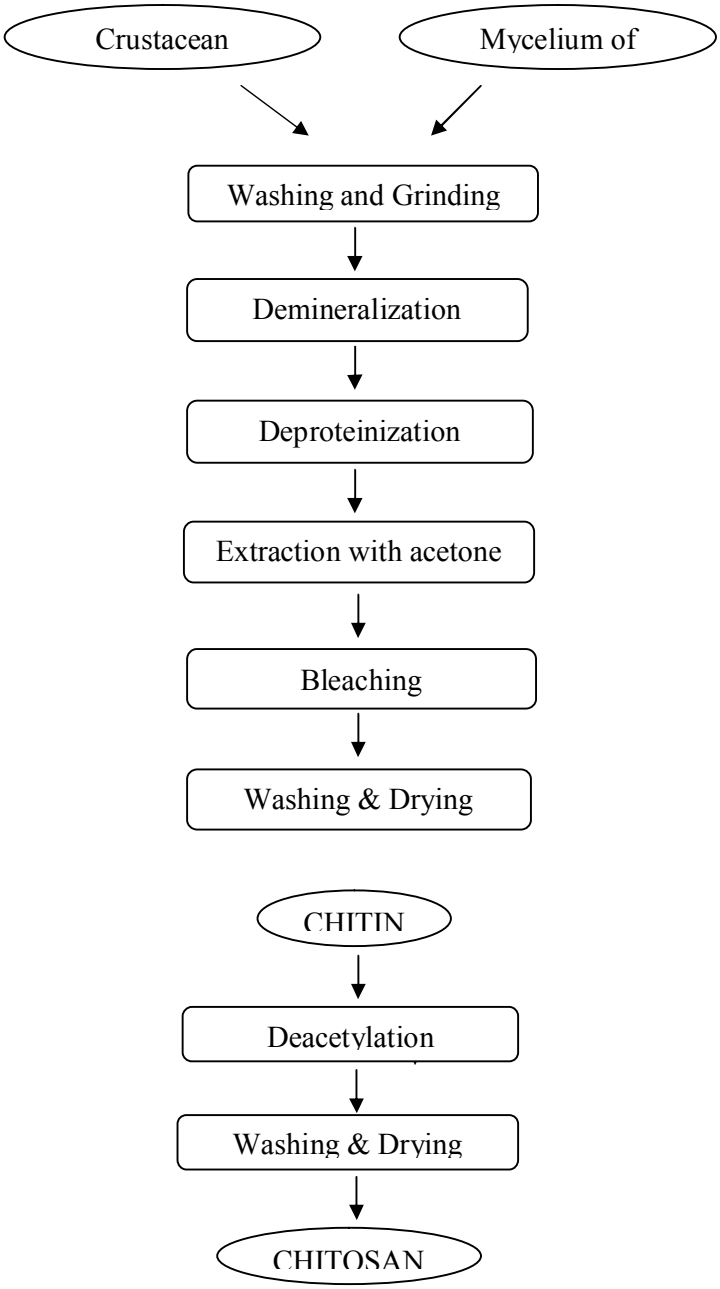

Fig: 1 Flow sheet for preparation of chitin & chitosan

EFFECT OF INCUBATION TIME ON ENZYME ACTIVITY

Enzyme activity with varying incubation time using SSF:

Enzyme activity of isolated strains (S1 & S2) and mutated strains (M1 & M2) were analyzed by varying incubation time using 0.1% urea as nitrogen source to determine the optimized metabolic environment for the production of β-galactosidase. Vogel's medium containing 0.1% urea inoculated with S2 gives the higher enzyme activity at fermentation time 48 hrs. The result indicates that 0.1% urea may contribute as a better nitrogen source for the production of β-galactosidase by isolated strain S2, using Vogel's medium. While Vogel's medium at fermentation time 48 hrs containing 0.1% ammonium sulphate may contribute as a second best nitrogen source for the production of β-galactosidase by isolated strain S1.

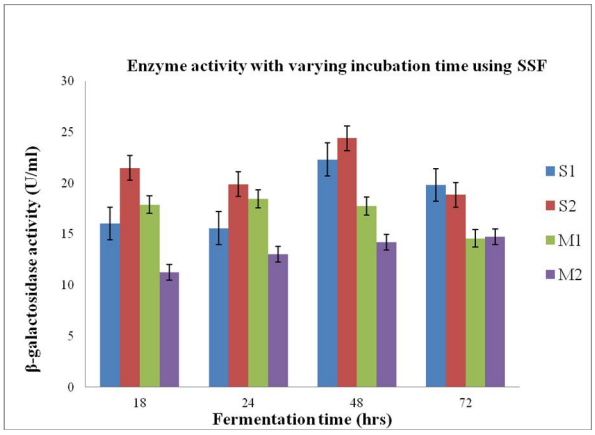

Fig 1: *Enzyme activity with S_1, S_2 & M_1, M_2 inoculated in Vogel's medium with different fermentation time using 0.1% urea as nitrogen source at pH 4.0*

Enzyme activity with varying incubation time using SmF:

Enzyme activity of isolated strains (S1 & S2) and mutated strains (M1 & M2) were analyzed by varying incubation time using glucose as carbon source & peptone as nitrogen source to determine the optimized metabolic environment for the production of β-galactosidase. YLK2Mg medium containing 0.1% glucose inoculated with S2 gives the higher enzyme activity at fermentation time 48 hrs. The result indicates that 0.1% glucose may contribute as a better carbon source for the production of β-galactosidase by isolated strain S2, using YLK_2Mg medium. While YLK_2Mg medium at fermentation time 48 hrs containing 0.1% glucose may contribute as a second best carbon source for the production of β-galactosidase by mutated strain M2.

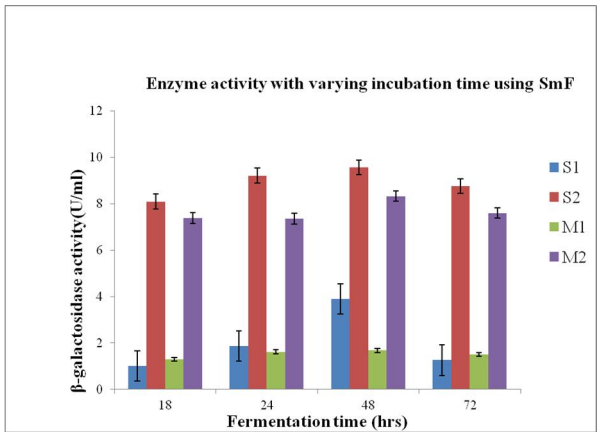

Fig 2: *Enzyme activity with S_1, S_2 & M_1, M_2 inoculated in YLK_2Mg medium with different fermentation time & glucose as carbon source & peptone as nitrogen source*

EFFECT OF R.P.M ON β-GALACTOSIDASE ACTIVITY:

Enzyme activity with varying rpm speed using SmF:

Enzyme activity of isolated strains (S1 & S2) and mutated strains (M1 & M2) were analyzed by varying rpm speed using glucose as carbon source to determine the optimized metabolic environment for the production of β-galactosidase. YLK2Mg medium containing 0.1% glucose inoculated with S2 gives the higher enzyme activity at 190 rpm speed. The result indicates that 0.1% glucose may contribute as a better carbon source for the production of β-galactosidase by isolated strain S2 at 190 rpm, using YLK2Mg medium through SmF. While YLK2Mg medium at 190 rpm speed containing 0.1% glucose may contribute as a second best carbon source for the production of β-galactosidase by mutated strain M2.

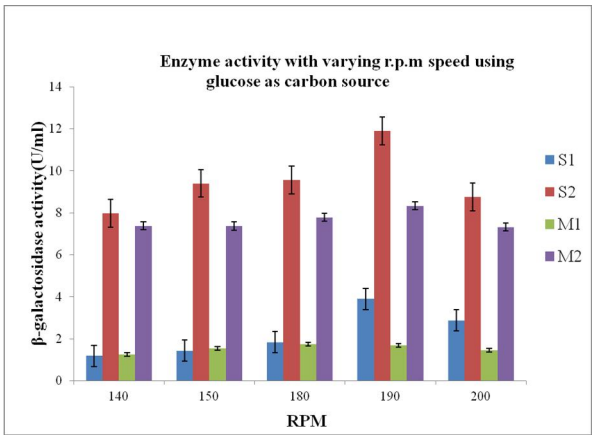

Fig 3: *Enzyme activity with S_1, S_2 & M_1, M_2 inoculated in YLK_2Mg medium with different fermentation time & glucose as carbon source & peptone as nitrogen source*

Enzyme activity with varying rpm speed using SmF:

Enzyme activity of isolated strains (S1 & S2) and mutated strains (M1 & M2) were analyzed by varying rpm speed using lactose as carbon source to determine the optimized metabolic environment for the production of β-galactosidase. YLK2Mg medium containing 0.1% lactose inoculated with S2 gives the higher enzyme activity at 190 rpm speed. The result indicates that 0.1% lactose may contribute as a better carbon source for the production of β-galactosidase by isolated strain S2 at 190 rpm, using YLK2Mg medium through SmF. While YLK2Mg medium at 180 rpm speed containing 0.1% lactose may contribute as a second best carbon source for the production of β-galactosidase by isolated strain S2.

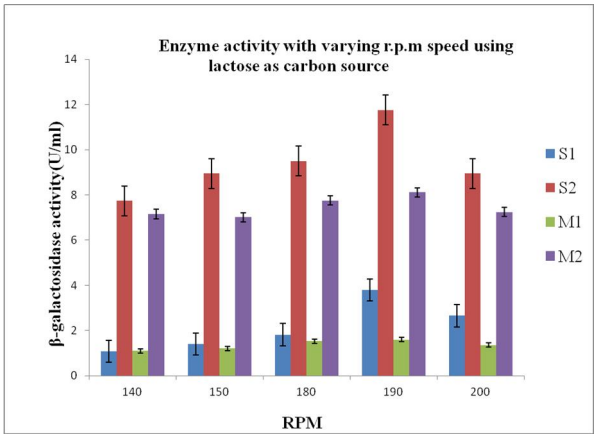

Fig 4: *Enzyme activity with S_1, S_2 & M_1, M_2 inoculated in YLK_2Mg medium with different fermentation times & lactose as carbon source, peptone as nitrogen source*

Enzyme activity with varying rpm speed using SmF:

Enzyme activity of isolated strains (S1 & S2) and mutated strains (M1 & M2) were analyzed by varying rpm speed using sucrose as carbon source to determine the optimized metabolic environment for the production of β-galactosidase. YLK2Mg medium containing 0.1% sucrose inoculated with S2 gives the higher enzyme activity at 190 rpm speed. The result indicates that 0.1% sucrose may contribute as a better carbon source for the production of β-galactosidase by isolated strain S2 at 190 rpm, using YLK2Mg medium through SmF. While YLK2Mg medium at 180 rpm speed containing 0.1% sucrose may contribute as a second best carbon source for the production of β-galactosidase by isolated strain S2.

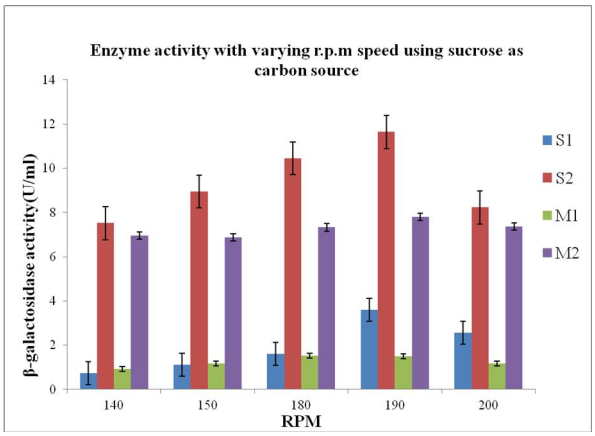

Fig 5: *Enzyme activity with S_1, S_2 & M_1, M_2 inoculated in YLK_2Mg medium with different fermentation time & sucrose as carbon source & peptone as nitrogen source*

Enzyme activity with varying rpm speed using SmF:

Enzyme activity of isolated strains (S1 & S2) and mutated strains (M1 & M2) were analyzed by varying rpm speed using starch as carbon source to determine the optimized metabolic environment for the production of β-galactosidase. YLK2Mg medium containing 0.1% starch inoculated with M2 gives the higher enzyme activity at 190 rpm speed. The result indicates that 0.1% starch may contribute as a better carbon source for the production of β-galactosidase by mutated strain M2 at 190 rpm, using YLK2Mg medium through SmF. While YLK2Mg medium at 140 rpm speed containing 0.1% starch may contribute as a second best carbon source for the production of β-galactosidase by isolated strain S2.

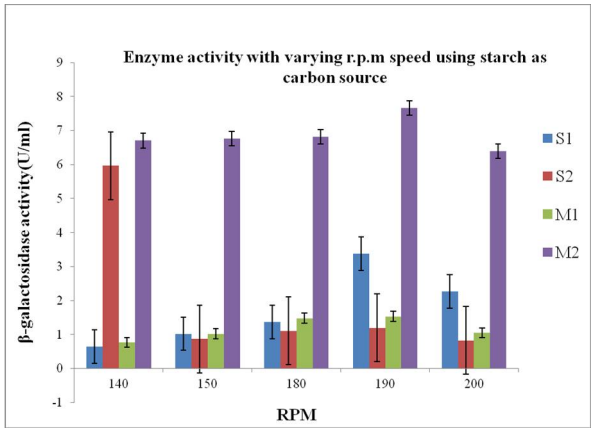

Fig 6: *Enzyme activity with S_1, S_2 & M_1, M_2 inoculated in YLK_2Mg medium with different fermentation time & starch as carbon source & peptone as nitrogen source*

EFFECT OF INDUCER ON ENZYME ACTIVITY

Enzyme activity with varying inducer concentration using SSF:

Enzyme activity of isolated strains (S1 & S2) and mutated strains (M1 & M2) were analyzed by varying inducer (IPTG) concentration using 0.1% urea as nitrogen source to determine the optimized metabolic environment for the production of β-galactosidase. Vogel's medium containing 0.1% urea inoculated with S2 gives the higher enzyme activity at 10^{-3} M. The result indicates that 0.1% urea may contribute as a better nitrogen source for the production of β-galactosidase by isolated strain S2 at 10^{-3} M, using Vogel's medium through SSF. While Vogel's medium at 10^{-3} M containing 0.1% urea may contribute as a second best nitrogen source for the production of β-galactosidase by mutated strain M2.

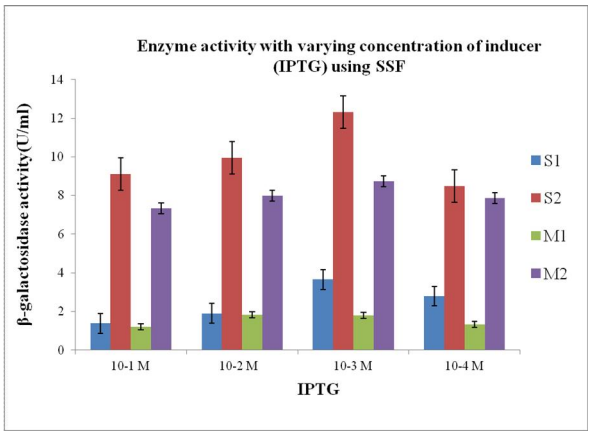

Fig 7: *Enzyme activity with varying concentration of inducer (IPTG) using SSF*

Enzyme activity with varying inducer concentration using SSF:

Enzyme activity of isolated strains (S1 & S2) and mutated strains (M1 & M2) were analyzed by varying inducer (glucose) concentration using 0.1% urea as nitrogen source to determine the optimized metabolic environment for the production of β-galactosidase. Vogel's medium containing 0.1% urea inoculated with S1 gives the higher enzyme activity at 3.0 gm of glucose as inducer. The result indicates that 3.0 gm glucose may contribute as a better inducer for the production of β-galactosidase by isolated strain S1, using Vogel's medium through SSF. While Vogel's medium at 3.0 gm glucose containing 0.1% urea may contribute as a second best inducer source for the production of β-galactosidase by isolated strain S2.

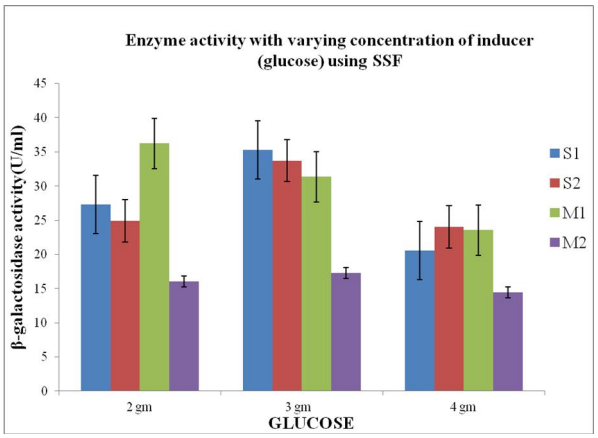

Fig 8: *Enzyme activity with varying concentration of inducer (glucose) using SSF*

Enzyme activity with varying inducer concentration using SSF:

Enzyme activity of isolated strains (S1 & S2) and mutated strains (M1 & M2) were analyzed by varying inducer (lactose) concentration using 0.1% urea as nitrogen source to determine the optimized metabolic environment for the production of β-galactosidase. Vogel's medium containing 0.1% urea inoculated with M2 gives the higher enzyme activity at 4.0 gm of lactose as inducer. The result indicates that 4.0 gm lactose may contribute as a better inducer for the production of β-galactosidase by mutated strain M2, using Vogel's medium through SSF. While Vogel's medium at 4.0 gm lactose containing 0.1% urea may contribute as a second best inducer source for the production of β-galactosidase by isolated strain S2.

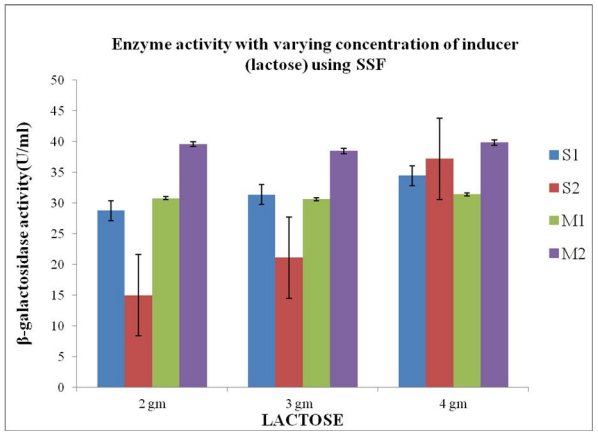

Fig 9: *Enzyme activity with varying concentration of inducer (lactose) using SSF*

Enzyme activity with varying inducer concentration using SmF:

Enzyme activity of isolated strains (S1 & S2) and mutated strains (M1 & M2) were analyzed by varying inducer (IPTG) concentration using YLK_2Mg medium to determine the optimized metabolic environment for the production of β-galactosidase. YLK_2Mg medium containing 10^{-3} M IPTG inoculated with S2 gives the higher enzyme activity. The result indicates that 10^{-3} M IPTG may contribute as a better inducer for the production of β-galactosidase by isolated strain S2, using YLK_2Mg medium through SmF. While YLK_2Mg medium at 10^{-3} M IPTG containing 0.1% glucose may contribute as a second best inducer source for the production of β-galactosidase by mutated strain M2.

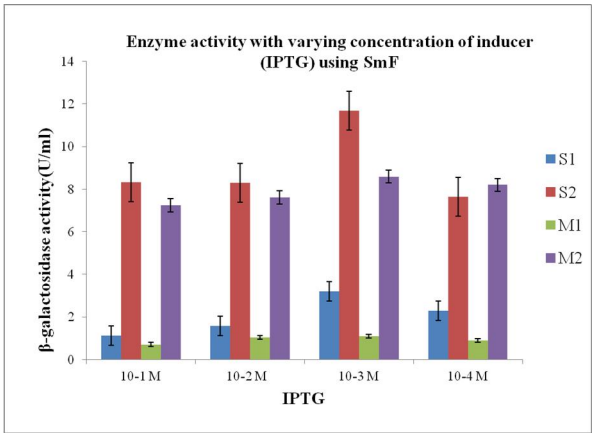

Fig 10: *Enzyme activity with varying concentration of inducer (IPTG) using SmF*

Enzyme activity with varying inducer concentration using SmF:

Enzyme activity of isolated strains (S1 & S2) and mutated strains (M1 & M2) were analyzed by varying inducer (glucose) concentration using YLK_2Mg medium to determine the optimized metabolic environment for the production of β-galactosidase. YLK_2Mg medium containing 3.0 gm glucose inoculated with S2 gives the higher enzyme activity. The result indicates that3.0 gm glucose may contribute as a better inducer for the production of β-galactosidase by isolated strain S2, using YLK_2Mg medium through SmF. While YLK_2Mg medium at 2.0 gm glucose may contribute as a second best inducer source for the production of β-galactosidase by mutated strain M2.

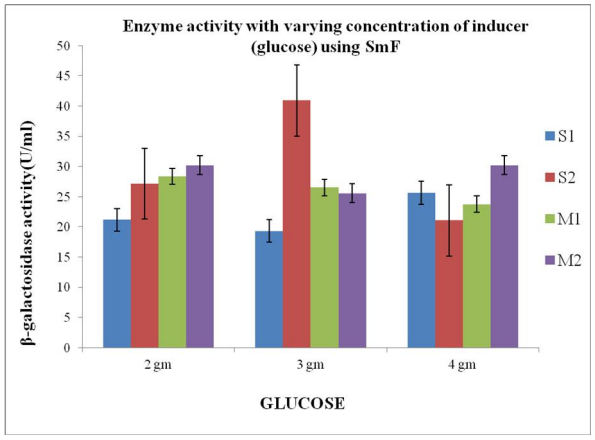

Fig 11: *Enzyme activity with varying concentration of inducer (glucose) using SmF*

Enzyme activity with varying inducer concentration using SmF:

Enzyme activity of isolated strains (S1 & S2) and mutated strains (M1 & M2) were analyzed by varying inducer (lactose) concentration using YLK_2Mg medium to determine the optimized metabolic environment for the production of β-galactosidase. YLK_2Mg medium containing 4.0 gm lactose inoculated with M2 gives the higher enzyme activity. The result indicates that 4.0 gm lactose may contribute as a better inducer for the production of β-galactosidase by mutated strain M2, using YLK_2Mg medium through SmF. While YLK_2Mg medium at 4.0 gm lactose may contribute as a second best inducer source for the production of β-galactosidase by isolated strain S1.

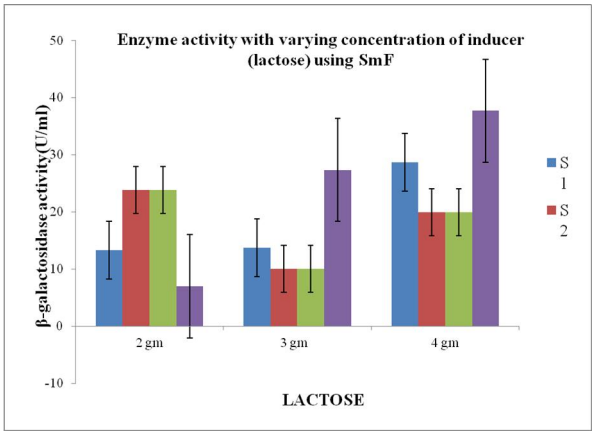

Fig 12: *Enzyme activity with varying concentration of inducer (lactose) using SmF*

EFFECT OF INHIBITOR ON ENZYME ACTIVITY

Enzyme activity with varying inhibitor concentration using SSF:

Enzyme activity of isolated strains (S1 & S2) and mutated strains (M1 & M2) were analyzed by varying inhibitor concentration using 0.1% urea as nitrogen source.

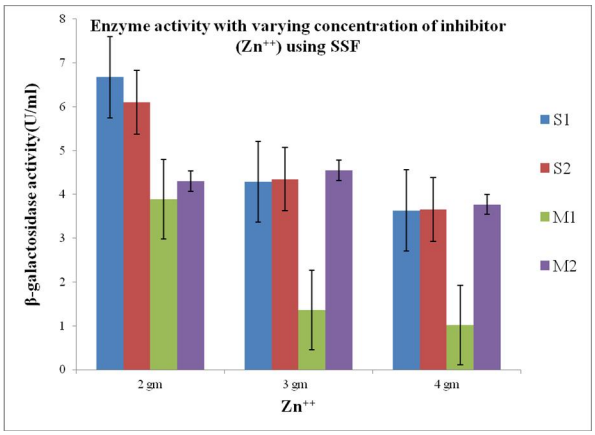

Fig 13: *Enzyme activity with varying concentration of inhibitor (Zn^{++}) using SSF*

Enzyme activity with varying inhibitor concentration using SmF:

Enzyme activity of isolated strains (S1 & S2) and mutated strains (M1 & M2) were analyzed by varying inhibitor concentration using YLK_2Mg medium.

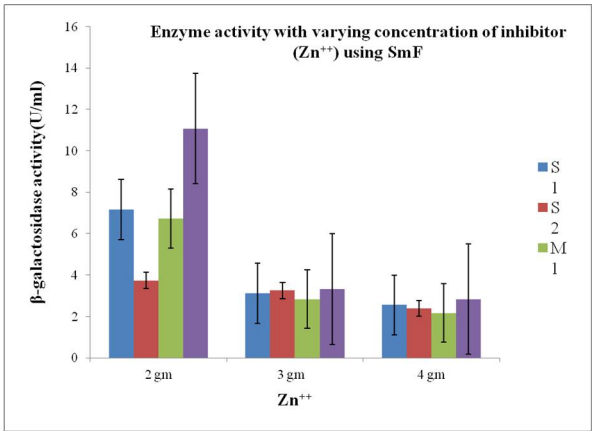

Fig 14: *Enzyme activity with varying concentration of inhibitor (Zn^{++}) using SmF*

EFFECT OF IMMOBILIZATION ON ENZYME ACTIVITY

β-galactosidase was immobilized on chitosan microsphere with 2, 4, 6 – trichloro – 1, 3, 5 –tri-azine as cross linker, and compared with that using glutaraldehyde. Chitosan with different molecular weight was used as material to immobilize β-galactosidase.

Effect of binding of enzyme β-galactosidase on chitin w. r. t time

Enzyme activity of isolated strain (S1) and mutated strain (M2) were analyzed by immobilization on chitin used as solid matrix. As the results obtained from the isolated strain (S2) & mutated strain (M1) were not very much satisfactory so therefore only the enzyme produced from the remaining two strains was immobilized.

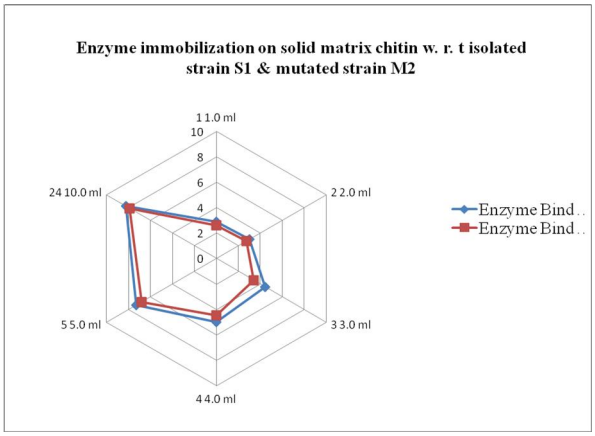

Fig 15: *Enzyme immobilization on solid matrix chitin w. r. t isolated strain S1 & mutated strain M2*

Effect of binding of enzyme β-galactosidase on chitosan w. r. t time

Enzyme activity of isolated strain (S1) and mutated strain (M2) were analyzed by immobilization on chitosan used as solid matrix.

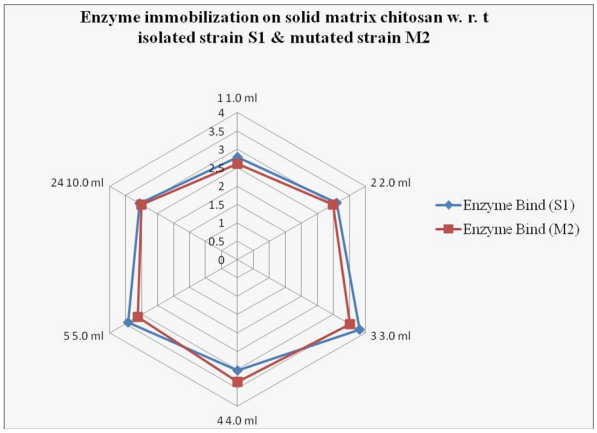

Fig 16: *Enzyme immobilization on solid matrix chitosan w. r. t isolated strain S1 & mutated strain M2*

Comparison of immobilization of enzyme β-galactosidase on chitin & chitosan w. r. t time

Enzyme activity of isolated strain (S1) and mutated strain (M2) were analyzed through immobilization on chitin & chitosan used as solid matrix.

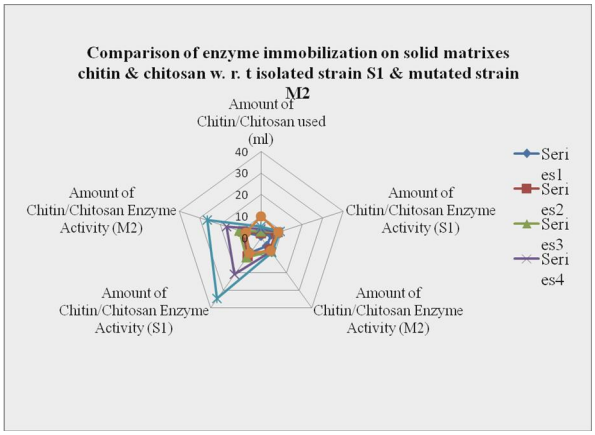

Fig 17: *Comparison of enzyme immobilization on solid matrixes chitin & chitosan w.r.t isolated strain S1 & mutated strain M2*

CONCLUSION

In dairy industry, β-galactosidase enzyme is rather used as a crude extract to prevent high cost of purification. Thus it is important to characterize the enzyme in crude extract of β-galactosidase producers. For this approach to be commercially successful, strains possessing high levels of β-galactosidase activity need to be identified.

In this study, we investigated the effect of carbon and nitrogen sources through SSF & SmF on β-galactosidase enzyme activity. We have also investigated the effect of pH on β-galactosidase activity through SSF & SmF mode of operations.

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