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Research article

A STUDY ON β-GALACTOSIDASE OF LACTOBACILLUS SP FROM MILK PRODUCTS AND ITS APPLICATIONS

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ABSTRACT: β-galactosidases enzyme have been used in the dairy industry for the improvement of lactose intolerance. The aim of the present study was to isolate β-galactosidase enzyme produced by isolated lactobacillus from milk and cheese. Isolated lactobacilli were cultured on MRS agar. Lactobacilli were identified by Gram staining and standard bacteriological and biochemical methods. Their ability to hydrolyze 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and O-nitrophenyl- β-Dgalactopyranoside (ONPG) was determined. β-galactosidase enzyme activity was also detected by Sodium Dodecyl Sulphate Gel electrophoresis (SDS-PAGE) method. The colonies that produced blue green color on X-Gal plates were lactobacillus with β-galactosidase enzyme which had ONPG positive results. By adding *Lactobacillus* producing β-galactosidase enzyme as probiotic to dairy products, could help lactose intolerant infants.

Keywords – β-galactosidase, *Lactobacillus*, MRS agar, X-Gal, ONPG, SDS-PAGE.

INTRODUCTION

The lactose- hydrolyzing enzyme, β-galactosidase (β-D-galactoside galacto hydrolase, E.C.3.2.1.23, trivially lactase) has long been accepted as an important enzyme for dairy industry. β-galactosidase catalyze two reactions: it catalyses hydrolysis of lactose, the milk sugar into glucose and galactose and in some cases β-galactosidase is able to catalyze transglycosylation reactions. In dairy industry β-galactosidase has been used to prevent crystallization of lactose, to improve sweetness, to increase the solubility of the milk product.

Lactic acid bacteria (LAB) that used as starters for production of dairy products are the main factors of fermentation and protection of fermentative foods and also have a significant role in texture and flavour of food products (Chammas et al., 2006). One of the glycosidases,is β-galactosidase enzyme that widely used in dairy industry and is produced by most lactobacilli

(Karasova et al., 2002; Corral et al., 2006; Nguyen et al., 2007). The enzyme hydrolyzes lactose, the main carbohydrate in milk, into glucose and galactose, which can be absorbed across the intestinal epithelium (Troelsen, 2005; Vasiljevic and Jelen, 2001; Heyman, 2006). β-galactosidase has two enzymatic activities: one is responsible for the hydrolysis of lactose and also cleaves cellobiose, cellotriose, cellotetrose and to a certain extent cellulose and the other, splits β-glycosides (Troelsen 2005; Heyman, 2006). Low activity of β-galactosidase causes digestive insufficiency, called lactose intolerance in most cases (Karasova et al., 2002; Vasiljevic and Jelen, 2001). The symptoms of lactose intolerance such as abdominal pain and diarrhea, nausea, flatulence, and or bloating after the ingestion of lactose or lactose containing food substances which can lead to decrease quality of life, and daily activities. Treatment is relatively simple by eliminating lactose from the diet or by using of supplemental β-galactosidase enzyme replacement (Vasiljevic and Jelen, 2001).

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The commercial enzymes used for lactose hydrolysis are β-galactosidase of diverse origins. Possible sources of the enzyme are: plants, animal organs, bacteria, yeasts (intracellular enzyme), fungi and moulds (extracellular enzyme). Among them bacterial sources are preferable because of ease of fermentation, high activities of the enzyme and good stability. The bacterial species currently used by the dairy industry which produced β-galactosidase enzyme belong to genera of *Lactobacillus* and *Bifidobacterium* (Fernandez et al., 1999; Xanthopoulos et al.,1999; He et al., 2008). Lactic acid bacteria (LAB) which constitute a diverse group of lactococci, streptococci, and lactobacilli have become a focus of scientific studies for three particular reasons: a) lactose maldigesters may consume some fermented dairy products with little or no adverse effects b) LAB are generally regarded as safe (GRAS) so the enzyme derived from them might be used without extensive purification. Also these bacteria have role in texture and flavor of dairy products, sausage and some other fermentative foods.

MATERIALS AND METHODS

Isolation of Bacteria from its Source

For isolation of bacteria,2g of cheese and 2g of milk was added to 5ml of MRS broth and incubated at 37°C for 24 hrs.Then 50 µl of them were spread onto MRS agar. Plates were incubated at 37°C for 48 hrs.

MRS Medium [de Man, Rogosa and Sharpe]

Identification of Isolated Bacteria

Bacteria were examined by Gram staining and identified by standard bacteriological and biochemical methods (Sneath et al; 1984).

Study of β-Galactosidase Production

X-Gal Substrate

One colony of isolated bacteria were grown on MRS agar plates containing 60 µl X-gal (5-bromo-4-chloro-2-indolyl-β-D-galactopyranoside 20mg/ml DMF) and 10 µl of IPTG (iso-propyl-thio- β-D galactopyranoside) solution as an inducer. Plates were incubated at 37°C for 24 hrs to 3 days. Colonies of β-galactosidase were identified as green in color.

ONPG Substrate

All bacteria were inoculated into tubes containing ONPG (O-nitrophenyl β-D-galactopyranoside) (0.5 ml) and 0.01 M sodium phosphate buffer (pH 7.0) (5 ml) and peptone water. Presence of yellow color was indicated as positive ONPG results for the present study.

Enzyme Extraction

Preparation of Cell Free Crude Extract from MRS Growth Medium (100 ML):

A total of 10 ml of culture was taken from the 100 ml MRS broth and harvested by centrifugation at 8000 rpm for 9 minutes at 9° C. After centrifugation, the pellet was washed with same amount of distilled water (10ml). The pellet was resuspended in 4.5 ml of 0.05M sodium phosphate buffer at pH 7 followed by vigorous vortexing to disperse the cells homogenously. The solution was labelled as "pellet solution".100 mg lysozyme was added into the tubes containing 4.5 ml pellet solution. After gentle mixing, this mixture was incubated at 37° C for 15 min. After 15 minutes of incubation at 37° C, 0.5 ml 4 M NaCl solution was added into the pellet solution. The solution was further incubated at 37° C for 50minutes. The solution was centrifuged at 8000 rpm for 9 minutes at 9° C. After the centrifugation, the supernatant was immediately transferred into eppendorf tubes. The supernatant was stored at 4°C. The present suspension was labelled as "cell-free crude extract".

Purification of Enzyme

Cold Acetone Precipitation

The sufficient amount of crude enzyme extract was taken and two fold of cold acetone was added. After gentle mixing, it is stored at -20°C for 20 minutes. Then the enzyme extract was centrifuged at 8000rpm for 40 minutes. The supernatant is discarded and protein pellet was dissolved in proper buffer (20Mm Tris-Hcl buffer, pH 7.2). The purified protein sample is stored at 4°C.

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ONPG Standard Curve

ONPG is an important compound as an indicator for β-galactosidase activity. In this assay, free ONPG at different dilutions level was used to construct ONPG standard curve. During construction of ONPG standard curve, known amount of ONPG with several dilutions were prepared. Each dilution gave a specific yellow color under the assay conditions with absorption peak at 420 nm.

During the enzymatic assay 1 ml of ONPG solution was mixed with 0.2 ml cell-free crude extract solution. Therefore during standard curve preparation 1 ml of appropriately diluted ONPG solution was mixed with 0.2 ml distilled water. Final molarity of ONPG was calculated from the equation at below

M1 * V1 = M2 * V2

M1: Initial molarity of ONPG solution

M2: Final molarity ONPG solution

V1: Initial volume of appropriately diluted ONPG solution

V2: Final volume of appropriately diluted ONPG solution

Assay of β-Galactosidase

The β-galactosidase activity was determined by using stopped assay protocol. In stopped assay, the reaction was allowed to proceed at one minute interval. Then, the reaction was stopped by addition of sodium carbonate. Sodium carbonate elevates the pH of the reaction medium up to 10; at this pH the enzyme was deactivated therefore no color formation occurs. Appropriate enzyme and substrate blanks were prepared.

The number of mole of ONP (O-Nitrophenol) liberated was determined from the standard curve of ONP. Units of the enzyme activity were expressed as the amount of enzyme required to release 1 μmoles of ONP per minute under the assay conditions. The extinction coefficient of dissolved O-nitrophenol at 420 nm was found to be 3.1703 ml μmol^{-1} $cm^{-1.}$

The rate of absorbance change (A420/min) was calculated as follows:

□(A420/min) = □A420/min(Enzyme reaction) - □A420/min(Blank).

Specific activity was calculated as follows (Baran 1996):

$$U/mg\ protein = \frac{\Delta A_{420/min}}{\varepsilon \times mg\ protein/mL\ reaction\ medium}$$

ε (extinction coefficient) of ONP: 3.1703ml μmol^{-1} cm^{-1}

DETERMINATION OF PROTEIN:

Protein concentration was determined by the method of Lowry et al using Bovine Serum Albumin as standard.

SDS-PAGE

The purified protein samples were analyzed by Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE). Gels were run for 4 h at 110 V. Protein bands were visualized by staining with Coomassie Brilliant blue R-250 (Dunn et al; 1993)

Enzyme Characterisation

Effect of Substrate Concentration (ONPG) on β- Galactosidase Activity

The purified enzyme extract solution was used to observe the effect of substrate concentration on β-galactosidase activity. Kinetic constants, Km and Vmax, of the β-galactosidase were determined by changing the ONPG substrate concentration.ONPG substrate concentration in the assay medium were 4.165 mM, 8.33 mM, 12.5 mM, 16.66 mM, 20.83 mM, 25 mM (in 0.05 M phosphate buffer at pH 7). Michaelis-Menten plot were constructed to calculate the Km and Vmax.

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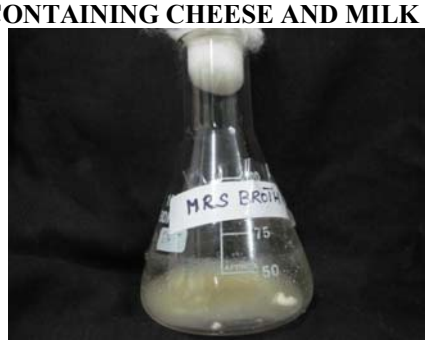
RESULTS and DISCUSSION

Isolation of Microorganism

The bacterial organism was isolated from MRS broth containing cheese and milk by spread plate method and colonies were observed successfully after 24 hr of incubation at 37°C.

MRS BROTH CONTAINING CHEESE AND MILK



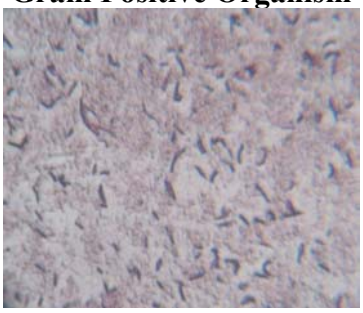
MRS AGAR PLATE WITH LACTOBACILLUS

Identification of Isolated Bacteria

Gram's Staining

On Gram's staining, purple color rod-shaped bacteria was observed under the microscopic examination (40X objective) indicates the presence of gram positive organism.

Gram Positive Organism



Biochemical Test Results for Confirmation of Lactobacillus Organism

Biochemical Test	Result
Mannose	Positive
Cellobiose	Positive
Sucrose	Positive
Maltose	Positive
Glucose (Acid)	Positive
Glucose (gas)	Weakly Positive
Fructose	Weakly Positive
Arabinose	Negative
Lactose	Negative
Sorbitol	Negative
Xylose	Negative
Mannitol	Negative
Rhamnose	Negative
Catalase	Negative
Nitrate reduction	Negative

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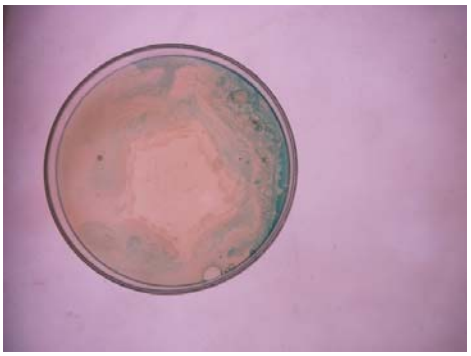
Study of β-Galactosidase Production
X-GAL Substrate

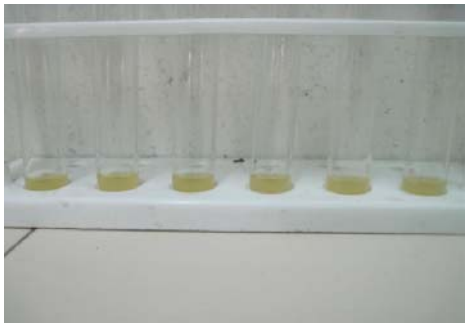
Spread plate Method



Streak Plate Method

ONPG Substrate

Before Inoculation of the Organism

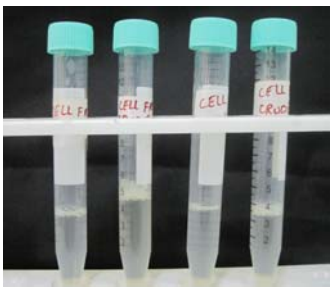


After Inoculation of the Organism

The heavy suspension of the organisms were inoculated in the tube containing ONPG broth and incubated at 37°C for 1-24 hrs. The production of yellow color indicates the presence of β-galactosidase.

Enzyme Extraction

The enzyme was extracted after centrifugation from the MRS growth medium. This enzyme solution was labelled as "cell-free crude extract" and stored at 4°C.



"CELL- FREE CRUDE EXTRACT"

Purification of Enzyme
Cold Acetone Precipitation

The crude enzyme extract was subjected to cold acetone precipitation. After centrifugation, the supernatant is discarded and protein pellet was dissolved in proper buffers (20Mm Tris-Hcl buffer, pH 7.2). The purified protein sample is stored at 4°C.

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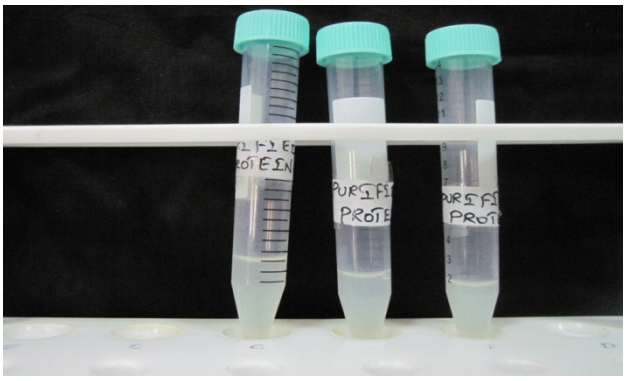

"PURIFIED PROTEIN SAMPLE"

ONPG Standard Curve

In this assay, free ONPG at different dilutions level was used to construct ONPG standard curve. During construction of ONPG standard curve, known amount of ONPG with several dilutions were prepared. Each dilution gave a specific yellow color under the assay conditions with absorption peak at 420 nm.

ONPG Standard Curve

S.NO	Concentration of ONPG solution in reaction medium (mM)	OD AT 420 nm
1	4.165	0.01
2	8.33	0.03
3	12.5	0.05
4	16.66	0.07
5	20.83	0.09
6	25	0.11

Stopped Enzyme Assay Method

SAMPLE	BLANK (ml)	TEST (ENZYME REACTION) (ml)
PURIFIED ENZYME SAMPLE	0.09	0.12

For stopped enzyme activity method, the rate of absorbance change (A420/min) was calculated as follows:

□ (A420/min) = □ A420/min (Enzyme reaction) – □ A420/min(Blank).

□ (A420/min) == 0.03

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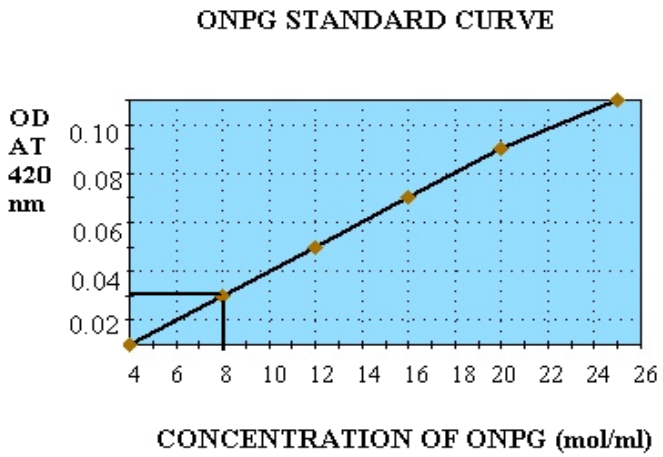
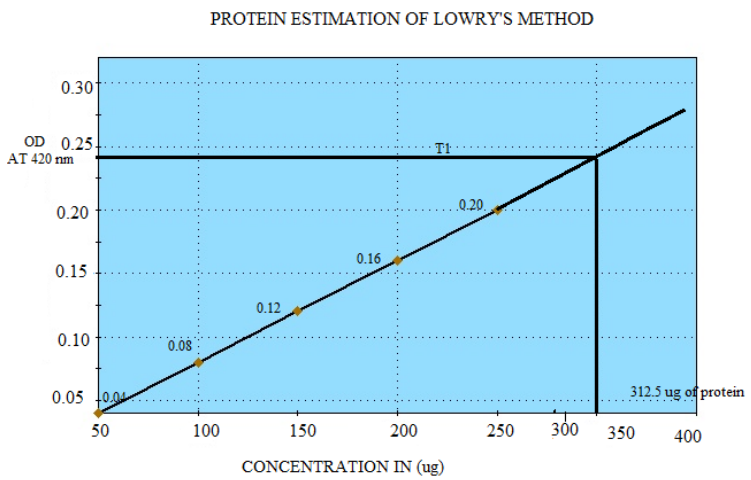
ONPG Standard Curve for Stopped Enzyme Assay



Estimation of Protein by Lowr'y Method



Estimation of Specific Activity of β-Galactosidase Enzyme by ONPG Standard Curve

Specific activity was calculated as follows (Baran 1996):

$$\text{U/mg protein} = \frac{\Delta A_{420}/\text{min}}{\varepsilon \times \text{mg protein/mL reaction medium}}$$

□ (extinction coefficient) of ONP: 3.1703ml µmol^{-1} cm^{-1}

$$\text{U/mg protein} = \frac{8\times10^{-3}\text{moles/min}}{3.1703\text{ml}\ \mu\text{mol}^{-1}\ \text{cm}^{-1} \times 31.25/7.5} \quad \text{U/mg protein} = 605$$

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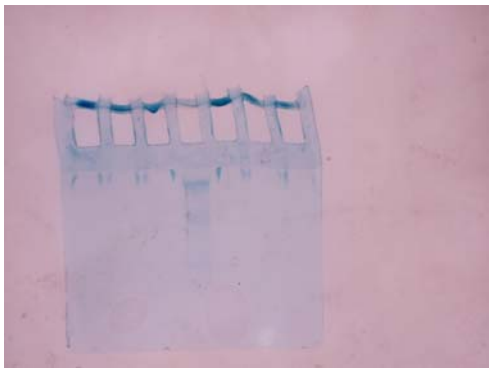
SDS-PAGE Results

The protein with a molecular weight of 116 kDa was observed in lactobacilli strain with the highest β-galactosidase enzyme value.

1 2 3 4 5 6 7



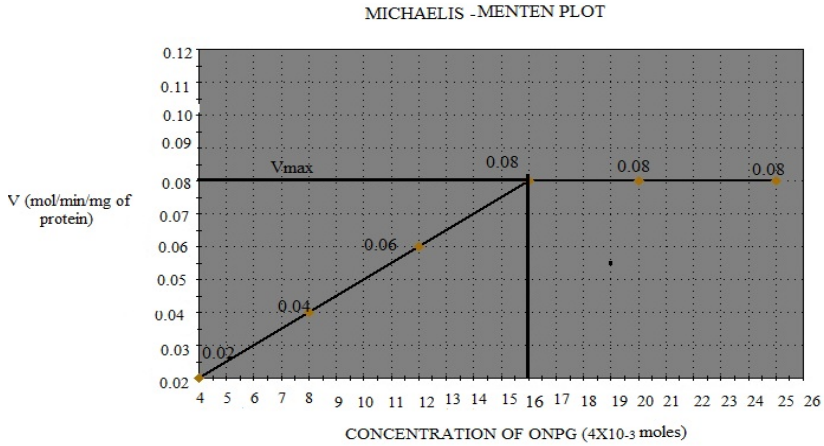
LANE 4 – PROTEIN MARKER (116 KDa)
LANE1 – β-GALACTOSIDASE SAMPLE

Effect of Substrate Concentration on β-Galactosidase Activity

The enzyme activity was measured at different concentrations of the substrate ONPG. The amount of the substrate used in the reaction medium were 4.165 mM, 8.33 mM, 12.5 mM, 16.66 mM, 20.83 mM, 25 mM (in 0.05 M phosphate buffer at pH 7). The enzyme β-galactosidase follows a parabolic Michaelis-Menten kinetics. The reaction rate was increased while the substrate (ONPG) concentration increased. Km and Vmax values of β-galactosidase were calculated from the reciprocal plots of substrate concentration versus reaction velocity. The Line weaver-Burk plot was linear, suggesting a simple Michealis-Menten kinetics. The Vmax was found as $16X10^{-3}$(mol/ min/ mg protein) and Km as $50X10^{-3}$ (moles/min).

S.NO	SUBSTRATE CONCENTRATION [S] (10^{-3} moles)	OPTICAL DENSITY AT 420 nm [V]
1	4	0.02
2	8	0.04
3	12	0.06
4	16	0.08
5	20	0.08
6	25	0.08

Michaelis-Menten Plot for β-Galactosidase of Lactobacillus



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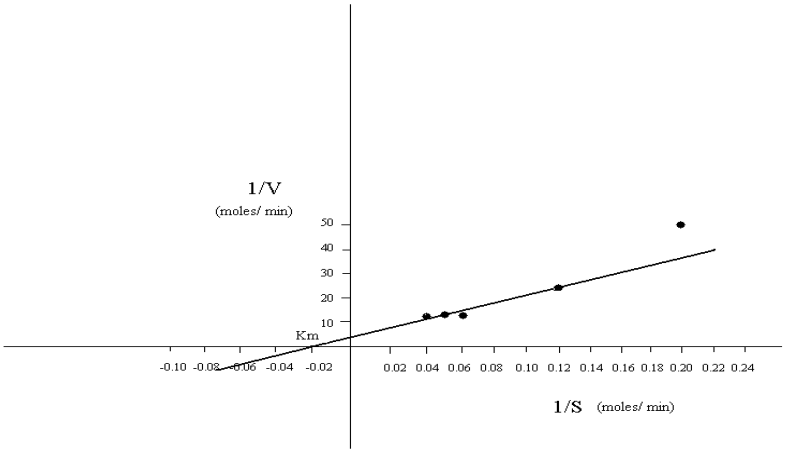
Lineweaver-Burk Reciprocal Plot of β-Galactosidase

S.NO	1/S	1/V
1	0.25	50
2	0.125	25
3	0.083	16.6
4	0.062	12.5
5	0.05	12.5
6	0.04	12.5

LB PLOT



The value of Km and Vmax was found to be $50X10^{-3}$(moles/min) and 16X10-3 (mol/ min/ mg protein)

Applications of Enzyme

Use of Lactase in Bread Making

To 10g of flour, 4ml of yeast suspension (7.5g 0f baker's yeast suspended in 95 ml of water) and 6 ml of a lactase (β-galactosidase) enzyme was added. The contents were mixed thoroughly with a spatula. After mixing to optimum consistency, the doughs were fermented at 30°C, after 10-20 mins doughess can be seen better in the bread containing enzyme than in control and texture of the bread is very soft in the test (bread containing the enzyme)

CONTROL: 10g flour + yeast suspension

TEXTURE OF BREAD – SOFT (TEST)

TEST: 10g flour + yeast suspension + lactase enzyme

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Use of Lactase in Ice Cream

Lactozym is a preparation of beta-galactosidase (lactase) which hydrolysis the disaccharide lactose to the sweeter-tasting monosaccharides glucose and galactose. Lactase is widely used in the manufacture of dairy products such as fermented milks, ice-cream, milk drinks and even lactose-reduced milk for cats.The ice cream is made from lactase-treated milk (or whey) .If the ice cream mix is treated with the enzyme, no lactose crystals are formed and the common defect 'sandiness', due to lactose crystallisation, will not occur. Hydrolysis of lactose also improves 'scoopability' and texture significantly, as well as making the ice cream more palatable to those who are lactose intolerant.

DISCUSSION

Lactose, the main sugar in milk and whey, and its corresponding hydrolase, β-galactosidase, have been the subject of extensive research during the past decade. Partly, this is because of the interesting possibilities of using low lactose or lactose free products.

In this study, the β-galactosidase enzyme was produced by isolated Lactobacillus strain in dairy products that is cheese and milk. *Lactobacillus* strains were selected for the present study.

Lactose intolerance has been recognized for many years as a common problem in many children and most adults throughout the world (Heyman, 2006). Therefore, by the addition of *Lactobacilli* producing β-galactosidase as probiotic to milk and cheese and other dairy products could help lactose intolerance symptoms.

In this study ONPG and X-gal were used as substrate for detecting β-galactosidase activity. Colonies growing on X-gal medium with bluish green color were regarded as bacteria containing β-galactosidase enzyme. It was observed that the cultures, which were incubated in MRS broth, could still produce β-galactosidase when they were transferred on the MRS agar. The bacteria were inoculated into tubes containing ONPG (O-nitrophenyl-β-D-galactopyranoside), 0.01 M sodium phosphate buffer and peptone water. Yellow color colonies were produced which indicates the positive result for ONPG.

From the present study, β-galactosidase enzyme was extracted from MRS broth. Since the enzyme is produced extracellularly by Lactobacillus sp, the extraction procedure is easy and the crude extract was subjected to purification for further studies.

It was reported that β-galactosidase enzyme is tetrameric enzyme which consists of identical subunits with a molecular weight of 116 kDa. From the present study, by using of SDS-PAGE method, an intensive 116 kDa protein band was observed in *Lactobacilli* with high values of β-galactosidase enzyme.

The Km and Vmax values of β-galactosidase enzyme from Lactobacillus were calculated by using ONPG as the substrate were found. From figure, the linearity of Line weaver-Burk double reciprocal plot was suggesting a simple Michealis- Menten kinetics. The value of Km and Vmax was found to be $50X10^{-3}$(moles/min) and 16X10-3 (mol/ min/ mg protein).

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