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Optimization of Lactase Production under Submerged Fermentation by *Lactobacillus sp.* KLSA 22

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ABSTRACT

Lactase yield was determined by inoculating Lactobacillus sp. KLSA 22 under optimized conditions of fermentation. Investigation results showed that maximum lactase activity (97.99 ± 02.38 IU) with 0.5% substrate (lactose in cheese whey) concentration at static condition. In addition, the studies of highest enzyme activity were observed at pH 6.5 and temperature 35°C at 48 h of incubation period. Under this optimal condition nitrogen source and metal ions used, yeast extract and $MgSO_4$ was found to favor the enzyme production respectively. The organism could be used for the commercial production of lactase for various applications in industrial scale.

Key words: *β-galactosidase, lactose, whey, enzyme production, Latobacillus sp. and ONPG.*

INTRODUCTION

Lactase (β-galactosidase, EC 3.2.1.23) hydrolyses lactose into its constituent glucose and galactose. This conversion is significant to nutrition and food technology since the major products of hydrolysis are: in combination, sweeter, more soluble, more easily fermented and directly absorbed from the mammalian intestine. These changes are cradle for the production of new products such as lactose-hydrolysed milk and whey[1]. Whey is the watery part of milk generated after curd formation as a by-product of cheese manufacturing which is produced in bulk quantity. The main solute is lactose in cheese whey at a concentration of about 4.5-5% and additional components are proteins, salts and vitamins that are present in less quantity. The low concentration of these components makes their recovery inefficient. As a result of its high organic content, dumping directly to the environment causes pollution problems. The resolution is bioconversion of whey into lactase has been performed in several countries[2]. In whey 4.8% content of lactose and relatively high levels of other nutrients that make it suitable as a microbial culture medium.

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Microbes are the producers of lactase and accomplished in using lactose as the sole source of carbon and energy[3].

Microbes are achieving considerable importance to their exploitation and suitability for the production of enzymes on account of development process to therapeutic and industrial importance[4]. Foremost, Lactic acid bacteria (LAB) are recognized in a wide diversity of microbes as regarded as safe sources[5]. The growth and enzyme production of the organism are strongly influenced by medium composition as a result optimization of media components and cultural parameters is the most important task in a biological process[6]. The selection of an inexpensive and easily accessible substrate accompanied by a suitable producer bacteria, optimization of culture conditions, and effective downstream processing are required to reduce the cost of enzyme preparation[7]. The present study describes the optimization of culture conditions on the production of lactase by *Lactobacillus sp.* KLSA 22.

MATERIALS AND METHODS

Culture retrieval for Production medium

The strain *Lactobacillus sp.* KLSA 22 was isolated and maintained in our Laboratory specifically used in this study[8]. During the study 1% of growing culture (1.2×10^8 cfu/ml) was inoculated in modified deMan Ragosa Sharpe (MRS) medium containing: Protease peptone 1%. Beef extract 1%. Yeast extract 0.55%. Triammonium citrate 0.2%. Sodium acetate 0.5%. K_2HPO_4 0.2%. $MgSO_4.7H_2O$ 0.01%. $MnSO_4.4H_2O$ 0.005%. Tween 80 0.1 ml and 2% Pure Lactose or Lactose in Cheese Whey was used as a rich source of carbon substrate in 250 ml Erlenmeyer flask, adjusted at pH of 6.5 and fermented till 72 h at 35°C afterwards samples were collected at every 24 h and processed for enzyme activity.

Enzyme Assay

Assay for the activity of lactase was performed as per the method described using lactose analog o-nitrophenyl-β-D-galactopyranoside (ONPG) as a chromogenic substrate prepared by dissolving 4 mg/ml of ONPG in 0.01M sodium phosphate buffer of pH 6.4[9,10]. 10 ml of fermented broth was collected and centrifuged at 10,000 rpm for 10 min at 4°C (GYROZEN 1580R). Afterward, the cell pellet was collected and washed with 10 ml of distilled water. Further, 0.5 ml of pellet was dissolved in 1.5 ml of 0.1M sodium phosphate buffer (pH 6.5) and cells were lysed by addition of 100 mg of lysozyme and the mixture was vortexed for 1 min. To this mixture 1 ml of ONPG was added and the resulting reaction mixture was incubated for about 15 min at 55°C[11]. This reaction mixture was stopped by adding 1ml of 1M Na_2CO_3 at room temperature for 1 min interval and processed for centrifugation at 10,000 rpm for 10 min at 10°C. Finally, the supernatant was measured at 420 nm in UV-visible spectrophotometer (SYSTRONICS AU-2700) and the concentration of ONPG was calculated by using standard o-nitrophenol (ONP) graph. One unit of lactase activity (IU) was defined as the amount of enzyme that liberates 1µmole of ONP per ml per min under assay conditions.

Optimization of Culture Conditions for Lactase Production

The effects of incubation time, pH and temperature decide a crucial task in the fermentation development process. These were carried out by cultivating the isolate at different time intervals (12-72 h) adjusting pH values (3-9) of MRS lactose broth using 1M HCl or 1M NaOH as required and incubated at different temperatures (20-40°C). Further, the same enzyme activity was determined.

Effect of pH and Temperature

The pH dependence was determined by using ONPG as a chromogenic substrate in a series of buffers at different pH values (3-9) i.e., 0.1M acetic acid-sodium acetate buffer (pH 3-6), 0.1M sodium phosphate buffer (pH 6.5-7.5) and 0.1M Tris HCl buffer (pH 8-9) at 37°C for 72 h. The activity of enzyme was measured at different pH after every 24 h. The temperature dependence was determined by incubating the bacterial culture at different temperatures ranging from 20-40°C at pH 6.5 for 72 h. Further, the activity of enzyme was measured after every 24 h against standard ONP graph.

Effect of Inoculum Size

The effect of different inoculum sizes ranged from 0.5 to 2% for the production of lactase carried out for 72 h at pH 6.5 and temperature 35°C. The samples were collected every 24 h and observed for the enzyme activity. Simultaneously, viable count of *Lactobacillus sp.* KLSA 22 was observed till 48 h of incubation period at 35°C.

Effect of Carbon Substrate Concentration

The two different rich source of carbon substrate i.e. Pure Lactose and Lactose in Cheese Whey were selected for the production of lactase in MRS medium at 0.5 to 2% concentrations. Fermentation was carried till 72 h at pH 6.5 and temperature 35°C moreover samples were analyzed for enzyme activity at every 24 h incubation period.

Effect of Agitation Speed

Effect of varying rate of agitation was investigated for lactase production at different substrate concentrations from 0.5 to 2% incubated at pH 6.5 and temperature 35°C in static condition as well as in orbital shaker at various speeds (50, 100, 150 and 200 rpm) and processed for enzyme activity.

Effect of Incubation Period

Incubation period shows the characteristics of the culture and is too based on the growth rate and enzyme production[12]. The effect of incubation period was determined by incubating production medium at different time intervals (12-72 h) at pH 6.5 and temperature 35°C. The samples were collected at every 12 h and further processed for enzyme activity.

Effect of Nitrogen Sources

The growth medium supplemented with different nitrogen sources i.e. beef extract, yeast extract, peptone and protease peptone were tested with 1% concentration at pH 6.5 and temperature 35°C for 48 h of fermentation thereafter enzymatic activity was detected.

Effect of Metal Ions

The effect of metal salts on lactase production is determined by adding different metal salts in the fermentation medium. The metal salts selected for present study were $ZnSO_4$, $CuSO_4$, $MgSO_4$ and $MnSO_4$ at 0.1% concentration at pH 6.5 and temperature 35°C for 48 h of fermentation and processed for enzyme activity.

RESULTS AND DISCUSSION

The strain *Lactobacillus sp.* KLSA 22 isolated and screened was used for the optimization studies[8]. During the enzyme assay, the enzyme substrate reaction mixture was stopped by the addition of Na_2CO_3 which elevates the pH of the reaction medium up to 11; at this pH enzyme inactivated and hence no color formation occurs. The production of yellow color indicates the presence of lactase due to the liberation of ONP, the yellow product of breakdown of ONPG, absorbs light at 420 nm. The amount of ONP present in the sample was calculated for lactase activity. If excess ONPG is added, the amount of ONP produced is proportional to the amount of lactase and time of the reaction (Fig. 1). The lactase activity was detected in cell pellet only but not in the supernatant, indicating it's an intracellular enzyme which is correlated with the findings of Mahalakshmi *et al.*,[13]. In similar way reported that intracellular lactase produced by *Bifidobacterium animalis* spp. *lactis* Bb12[14] and *L. delbrueckii* spp. *bulgaricus* ATCC 11842[15] and also *L. fermentum* K4[16] in contrast with extracellular lactase produced by *Bacillus licheniformis* ATCC 12759[12].

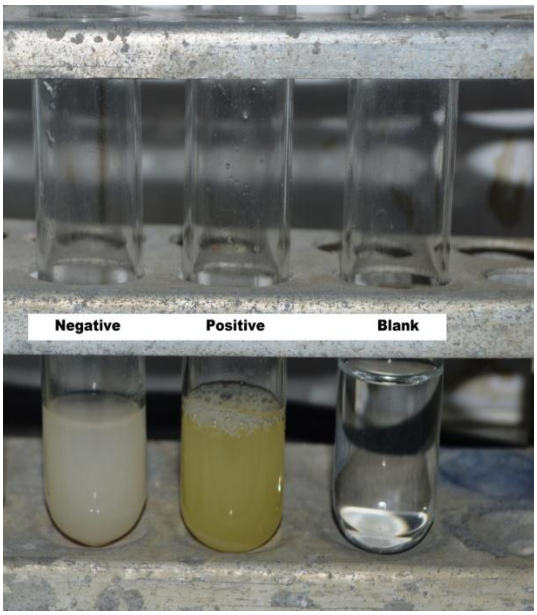

Fig. 1: Production of lactase by *Lactobacillus sp.* KLSA 22

Effect of pH and Temperature

The highest enzyme activity at pH 6.5 was 89.94 IU obtained (Fig. 2), which was also favorable pH for growth of *Lactobacillus sp.* KLSA 22[8]. However, the maximum lactase production by *L. amylophilus* GV6 was at pH 6.5 was reported[13]. In fact, lactase from *L. delbrueckii* spp. *bulgaricus* ATCC 11842 showed its maximum activity at pH 6.8 was reported[14]. On the other hand, Natarajan *et al.*, and Akcan also reported that lactase production was optimum at pH 7[11,12]. Maximum production of lactase at temperature of 35°C was 90.05 IU obtained. The enzyme production was steadily increased with increasing temperature up to 35°C, and later declined (Fig. 3). Similar findings of Natarajan *et al.*, reported that highest enzyme production at temperature of 35°C with *Bacillus* sp[11].

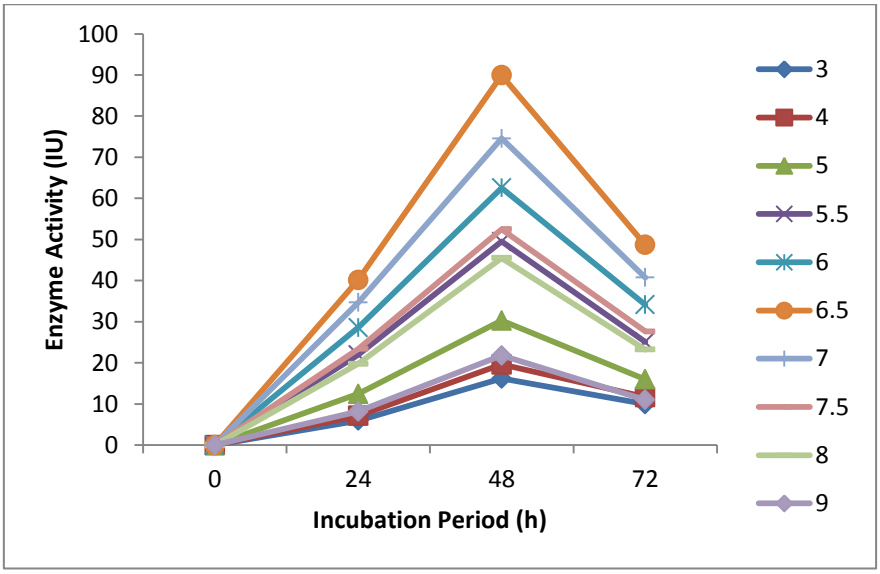

Fig. 2 Effect of pH on Lactase Production

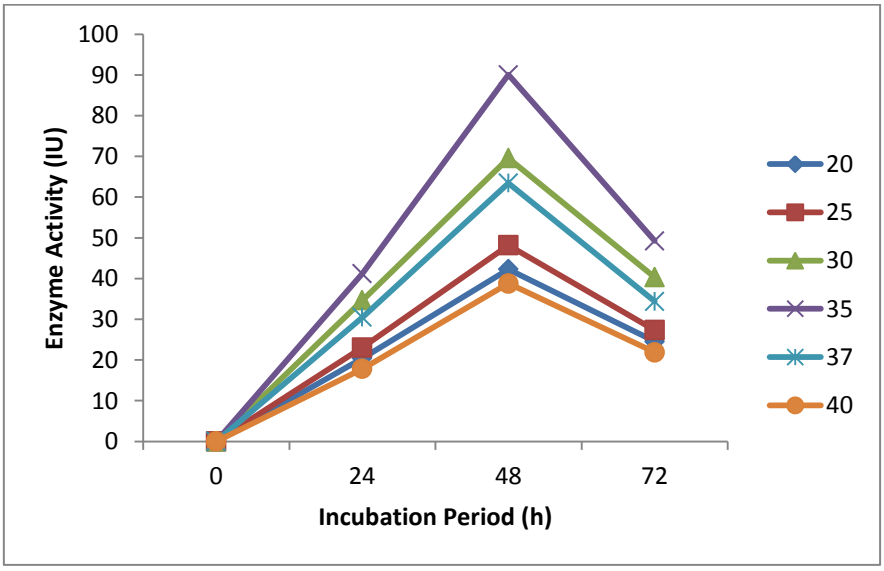

Fig. 3: Effect of Temperature on Lactase Production

Effect of Inoculum Size

Enzyme production varied with percentage of inoculum and the maximum enzyme production was 91.49 IU obtained with 1% inoculum of *Lactobacillus sp.* KLSA 22 at 35°C for 48 h. The viable cell count (colony forming unit, cfu/ml) showed that increased over incubation period up to 48 h. However increase of inoculum level from 1 to 2% showed a marginal decrease in lactase production but there was increase in cell count (Fig. 4 and Table 1). Similar observation on higher enzyme production with 1% inoculum of *L. amylophilus* GV6 was reported[13].

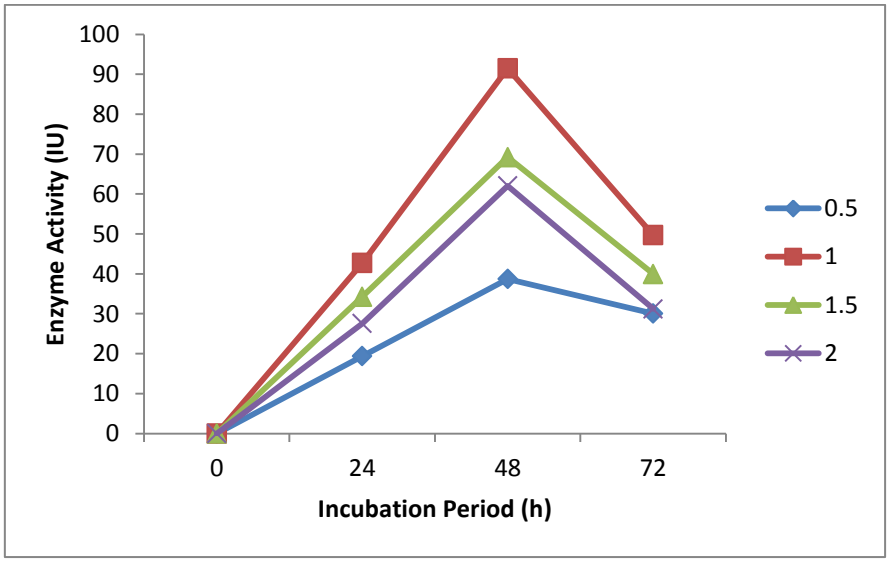

Fig. 4: Effect of Inoculum Size on Lactase Production

Table 1 Viable Count of *Lactobacillus sp.* KLSA 22 at variable Inoculum size at different incubation period

Inoculum size (ml)	**Cell count, cfu/ml**			
	12 h	24 h	36 h	48 h
0.5	2×10^6	16×10^6	34×10^6	86×10^8
1.0	8×10^6	27×10^6	61×10^6	1.2×10^8
1.5	12×10^6	32×10^6	83×10^6	1.6×10^8
2.0	22×10^6	45×10^6	97×10^6	1.8×10^8

Effect of Substrate Concentration

On supplementation of Pure Lactose and Lactose in Cheese Whey were tested as carbon substrates to evaluate the enzyme production by Submerged Fermentation (SmF). The maximum enzyme production obtained was 94.33 IU with 0.5% lactose in Cheese Whey at 48 h of fermentation thereafter production decreased (Table 2).

Table 2 Effect of Substrate Concentration at different incubation period on Lactase production

Substrate Concentration (%)	**Enzyme Activity (IU)**		
Pure Lactose	24 h	48 h	72 h
0.5	46.49	91.83	50.94
1.0	40.77	88.55	49.94
1.5	38.72	79.77	46.83
2.0	28.94	69.83	39.88
Lactose in Cheese Whey			
0.5	46.72	94.33	51.49
1.0	40.83	89.61	50.05
1.5	39.27	80.38	47.33
2.0	29.77	70.44	40.22

Table 3 Comparison between different organisms on lactase production

Producing Organisms	Concentration of Lactose (%)	Source of Substrate	Lactase Production
L. delbrueckii ssp. bulgaricus NIAI B-6[17]	2	Pure Lactose	844 U/ml
L. delbrueckii ssp. bulgaricus[18]	1	Pure Lactose	867-1966 U/ml
L. amylophilus GV6[13]	0.9	Pure Lactose or Lactose in Cheese Whey Permeate	2600-2700 U/ml
Lactobacillus sp. KLSA 22 (present study)	0.5	Pure Lactose or Lactose in Cheese Whey	97.99 ± 02.38 IU

Similarly, Honda *et al.,* reported that higher enzyme production (844 U/ml) with 2% pure lactose by *L. delbrueckii ssp. bulgaricus* NIAI B-6 from human faeces, whereas Gheyatanchi *et al.,* reported that *L. delbrueckii ssp. bulgaricus* and *L. casei* with 1% pure lactose produced highest enzyme activity (867-1966 U/ml)[17,18]. But, in contrast with 0.9% lactose in Cheese Whey Permeate the highest enzyme production (2600-2700 U/ml) by *L. amylophilus* GV6 was reported[13]. These finding correlates with the present study and that lactase by *Lactobacillus sp.* KLSA 22 isolated from cheese whey are good at lactase production even in low substrate concentration (Table 3).

Effect of Agitation Speed

The maximum lactase production was 97.99 IU observed under static condition at 0.5% lactose in cheese whey substrate concentration (Table 4). The enzyme production gradually declined, as the agitation speed increased, this finding correlated with Mahalakshmi *et al.,* also reported that highest lactase production by *L. amylophilus* GV6 at static condition[13].

Table 4 Effect of Agitation Speed at different substrate concentration on Lactase production

Substrate Concentration (%)	Enzyme Activity (IU)				
	Static	50 rpm	100 rpm	150 rpm	200 rpm
0.5	97.99	93.99	84.83	78.66	62.16
1.0	90.22	88.66	82.88	77.16	60.99
1.5	83.99	79.27	76.94	70.99	56.11
2.0	79.22	76.05	71.49	66.55	49.72

Effect of Incubation Period

The enzyme production gradually increases with the increasing period of incubation up to 72 h. It was found that highest lactase activity (95.61 IU) shown at 48 h of incubation period (Fig. 5). Similar observations on maximum β-galactosidase production at 48 h incubation period by *Bacillus* sp., *L. acidophilus* and *Bacillus licheniformis* was reported respectively[11,19,20].

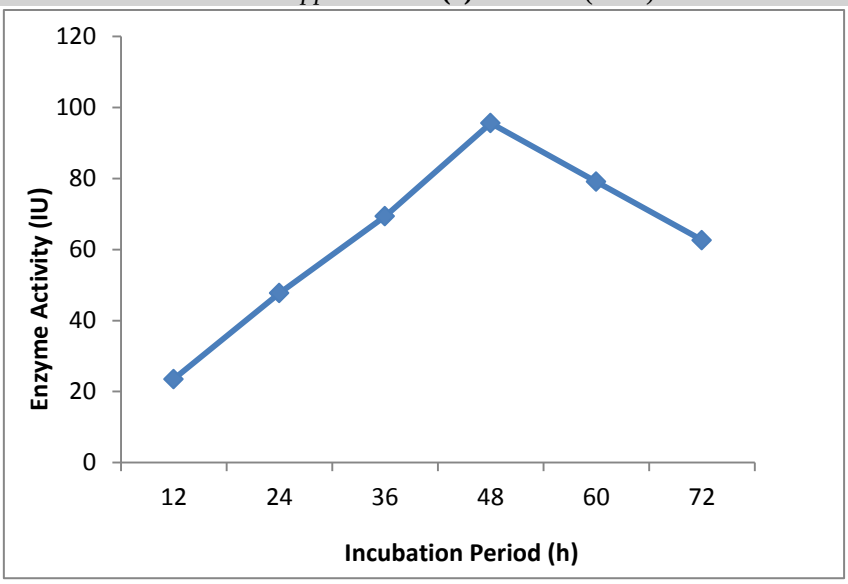

Fig. 5: Effect of Incubation Period on Lactase Production

Effect of Nitrogen Sources

Nitrogen sources can affect the microbial production of lactase[21,22]. The results revealed that yeast extract utilized as the sole source of nitrogen as it gave highest enzyme activity (89.94 IU) as compared to other nitrogen sources (Fig. 6). Hsu *et al.*, reported that yeast extract essential for lactase production, while casein, peptone and beef extract repressed lactase formation[22].

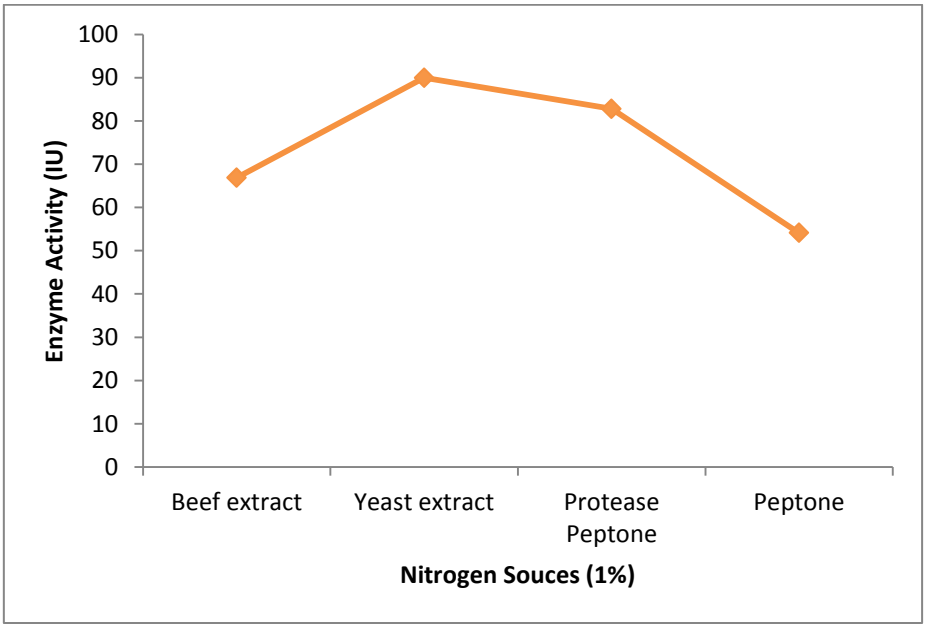

Fig. 6: Effect of Nitrogen Sources on Lactase Production

Effect of Metal Ions

The highest lactase production was 62.44 IU obtained at 48 h when the fermentation medium supplemented with $MgSO_4$ (Fig. 7). This indicate essential of Mg^{+2} for the stabilization of the enzyme. Rao and Dutta reported that the positive effect of Mg^{+2} and Mn^{+2} on lactase production[23].

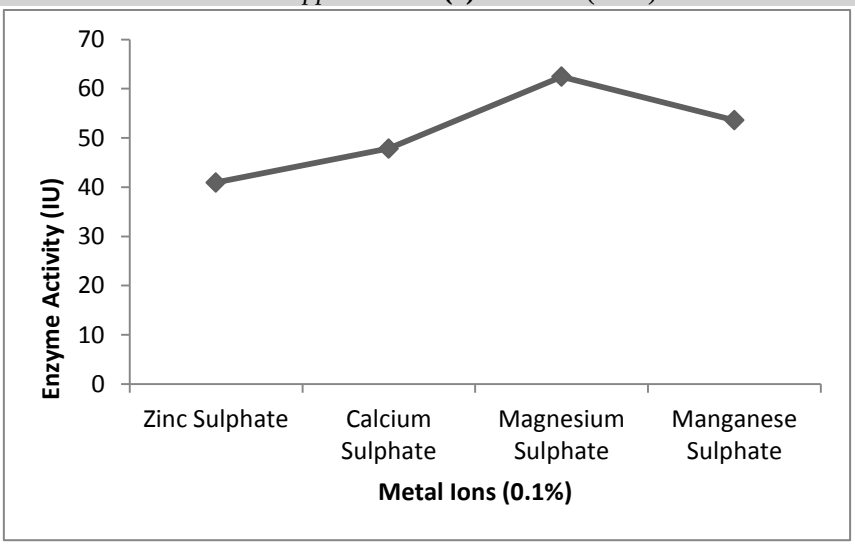

Fig. 7: Effect of Metal ions on Lactase Production

CONCLUSION

This study revealed that the strain *Lactobacillus sp.* KLSA 22 isolated from cheese whey be the best potential source of lactase, as it is utilized dairy waste cheese whey as a carbon substrate for the enhanced enzyme production at low-cost. This suggests that it can be used in industrial scale for a variety of applications in dairy industry.

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