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OPTIMIZATION AND IMMOBILIZATION OF *ESCHERICHIA COLI* PRODUCING β GALACTOSIDASE FROM DAIRY INDUSTRY

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ABSTRACT

An isolated strain of *Escherichia coli* MH2 was cultured and optimized for β galactosidase production. The Production medium was supplemented with lactose, peptone and beef extract. Several carbon sources were added to the medium. The bacterial culture utilized these carbon sources for β galactosidase production. Out of these, Xylose served as best carbon source, followed by glucose, lactose, sucrose, starch, fructose, dextrose and inositol. Among the various nitrogen sources used in this study, Yeast extract was found to be the best. Certain parameters involving incubation time, pH and temperature were studied separately. Maximum β galactosidase production was obtained at 24 hours, initial pH 7.0 and temperature 37° C. Various metal ions and natural substrates were optimized and their effects were studied. Ca^{2+}, Cu^{2+}, Na^{+} and lactose increased the production of β galactosidase. Immobilization of crude enzyme by calcium alginate method was investigated. Both free and immobilized enzyme exhibited the same pH 7.0 as optimum whereas temperature optima of immobilized enzyme was 40° C and 37° C for free enzyme. There was no loss in activity of immobilized β galactosidase till 25 days whereas free enzyme was stable for only 15 days. Immobilized enzyme was significantly more

stable against the free enzyme. β galactosidase from *Escherichia coli* MH2 strain holds promise for manufacturing lactose free dairy products at low cost.

Keywords: β galactosidase, *Escherichia coli* MH2, Lactase deficiency, optimization, immobilization

INTRODUCTION

β galactosidasealso known as lactase (EC 3.2.1.23) catalyzes the hydrolysis of lactose to glucose and galactose. β galactosidase is used to mitigate the problem of lactose intolerance, the inability to fully digest sugar (lactose) in dairy products by individuals who are deficient in lactase [1]. β galactosidase has been widely used in industrial application. In dairy industry, β galactosidase has been used to prevent crystallization of lactose, to improve sweetness and to increase the solubility of milk products [2]. β galactosidase can be obtained from various sources such as plants, animals and microorganisms [3]. Compared to animal and plant sources, the microbial enzyme is produced at higher yields and is more technologically important because it offers various advantages such as easy handling, higher multiplication rate, and high yield [4]. Commercial sources of β galactosidase are *Kluyveromyces lactis* and *Kluyveromyces marxianus* and moulds such as *Aspergillus niger* and *Aspergillus oryzae* [5, 6] and bacterial cultures are *Bacillus coagulans*, *Bacillus stearco thermophillus*, *Bacillus circulans*, *Escherichia coli*, *Lactobacillus bulgaricus*, *Lactobacillus thermophile* [7].

The activity and stability of the enzyme is influenced by the type of strain, cultivation conditions (temperature, pH, aeration, agitation, incubation time etc.) and the growth medium composition (particularly carbon and nitrogen sources) [8, 9, 10]. Therefore, attempts were made to increase the productivity by optimizing parameters with emphasis on carbon and nitrogen source, metal ions, pH, incubation time and temperature by using raw milk as substrate. The present study describes the effects of culture conditions on the production of β galactosidase by an isolated strain *Escherichia coli* MH2.

Immobilized enzymes are more favorable than the free enzymes, as they offer the possibility of continuous flow processing so that easy regeneration of the enzyme and low cost operation can be achieved [11]. Moreover, the soluble enzymes are normally used for batch processes while the immobilized enzyme lends itself to continuous operation [12, 13]. Thus, immobilization of enzymes has been a

widely employed technique in industrial applications due to enhanced stability, reusability, selectivity towards non natural substrates thereby making immobilization process cost effective and viable [14,15]. The present study deals with the immobilization of raw milk β galactosidase by calcium alginate immobilization method. The activity of free and immobilized β galactosidase was compared against parameters such as pH, temperature, incubation time and storage stability.

MATERIALS AND METHODS

Escherichia coli MH2 culture was isolated from raw milk of Khurana sweets and dairy, Mohali (Punjab). The cultures were maintained on nutrient agar plates and sub cultured regularly [16].

Effect of incubation time, temperature and pH on β galactosidase enzyme

The effect of incubation period, temperature and pH on β galactosidase production was investigated by cultivating the organism at different incubation time periods ranging from 6 to 60 hours, different temperatures (20°-70° C) and a pH range of 4 to 10. The organism was incubated and the β galactosidase activity was determined.

Effect of different substrates on β galactosidase enzyme

Different substrates of β galactosidase were used in the production medium. Wheat bran, rice bran, lactose, wheat flour, potato starch were used to perform enzyme assay in order to find out the optimum substrate in production medium for β galactosidase activity.

Effect of carbon and nitrogen sources on β galactosidase enzyme

Different carbon sources such as glucose, lactose, sucrose, starch, xylose, fructose, dextrose and inositol were employed to study their effect on β galactosidase production. All the carbon sources were supplemented at 1% concentration in the production medium.

Similarly nitrogen sources like yeast extract, beef extract, peptone, casein, urea and ammonium sulphate were employed to study the effect on β galactosidase production.

The bacterial isolate MH2 was grown in production medium containing different carbon and nitrogen sources at 37 °C for 24 h. The supernatant was used to perform the β galactosidase assay. The effect of carbon and nitrogen sources on the growth of bacterial isolate was studied by measuring the absorbance at 420 nm.

Effect of metal salts on β galactosidase enzyme

Different salts of metal ions ($CuSO_4$, $ZnSO_4$, $CaCl_2$, $FeSO_4$, NaCl, $MgSO_4$) at different

concentrations (0, 2, 4, 6, 8, 10 mM) were added and incubated with the enzyme extract and then enzyme assay was performed to determine the β galactosidase activity of enzyme.

Immobilization of Bacterial isolate MH2 by calcium alginate

A 3 % solution of sodium alginate was prepared by slowly adding alginate in 0.1 M phosphate buffer, pH 7.0 at 37 ° C. The β galactosidase enzyme was then added (0.1 mg/ml) to sodium alginate solution. After this, the alginate enzyme mixture in 20 ml syringe was loaded and the loaded mixture was dropped from constant height into 500 ml of chilled 0.2 M calcium chloride, with constant swirling on a magnetic stirrer. The enzyme got entrapped in a cage of calcium alginate in the shape of a bead. The beads were washed with buffer to remove excess calcium chloride solution and were stored in phosphate buffer at 4° C.

Comparative study of immobilized state of β galactosidase enzyme with its free state

Thermo stability

To determine optimum temperature, 6 free and immobilized enzymes were incubated at different temperatures (20° C to 70° C) for 30 min before the activity was measured. At specified time intervals, gel beads were removed and the activity was measured under standard assay conditions.

Optimum pH

To determine the optimum pH for β galactosidase activity of free and immobilized enzyme, the enzyme reaction was carried out using buffer of different pH values ranging from 4.0 to 10. Then, the enzyme assay was performed at standard conditions.

Incubation time

Effect of incubation time of enzyme reaction for both free and immobilized enzyme was determined by varying the time course from 5 to 30 min.

Storage stability

Both the free and the immobilized β galactosidase enzymes were stored at 4° C. The β galactosidase activity for both free and immobilized state was determined on intervals of 1, 5, 15, 20, 25 and 30 days under standard conditions.

RESULTS AND DISCUSSIONS

Effect of incubation time, pH and temperature

Incubation time period of the strain MH2 was optimized for maximum β galactosidase production. β galactosidase activity was determined at 0 h, 6 h, 12 h, 24 h, 36 h, 48 h and 60 h of incubation time period. Production ranged from 45.06 to 226.62

U/mg and the highest activity was recorded after 24h incubation period and the activity started to decline after 48 h (Figure 1a). Natrajan *et al* [18] reported β galactosidase producing BPTK4 isolated from the dairy effluent and the characterization of the enzyme and optimization of the production medium were carried out for the maximum production and activity of β galactosidase. Maximum production of enzyme was obtained when the medium was incubated for 48 hours and showed profound influence on the activity of enzyme (0.20 U/ml) at 48 hours.

Different pH values ranging from 4.0 to 10 of production medium were studied at their optimized temperature. Maximum β galactosidase activity of 305.63 U/mg was achieved at pH 7.0. Basic pH (7-9) was better for β galactosidase production as compared to acidic pH. At pH 4, β galactosidase activity was low of 94.69 U/mg and decreased by ~ 90 % of its maximum activity (Figure 1b). Maity *et al* [19] reported the β galactosidase was active at broad range of pH 3-10 and the maximum activity was observed at pH7. Similar results have been reported for several β galactosidase sources [20,21].

Different temperatures ranging from 20° C to 70° C were studied to optimize the temperature for β galactosidase activity. Study of temperature revealed that β galactosidase activity increased upto 37° C (Figure 1c) and started to decline above 37° C for the isolate MH2. Similarly, Maity *et al* [19] reported the intracellular β galactosidase enzyme of BS1 was active in a broad range of temperature 10-90° C and showed maximum activity of 39.17 U/mg at 40° C and a gradual decrease in the activity was observed at higher temperature.

Effect of different substrates

Different substrates such as wheat bran, rice bran, wheat flour, lactose, potato starch (1%) were used in the production medium with determined optimum pH and temperature. Maximum β galactosidase activity 331.45 U/mg was obtained on lactose substrate whereas potato starch and rice bran showed very low β galactosidase activity of 55.19 U/mg and 61.2 U/mg respectively. Thus, maximum β galactosidase acitivity achieved by lactose by isolate MH2 (Figure 2). Natrajan *etal* [18] reported the β galactosidase producing BPTK4 isolated from the dairy effluent and studied the effect of natural substrates such as wheat bran, wheat flour and rice bran and the medium was optimized for the maximum production of the enzyme and found that wheat bran showed a relatively high production of enzyme 0.17 U/ml and the production rate

was slightly decreased by other substrates. Similarly, Akcan [17] reported that when the production medium was supplemented with rice flour, maximum production of β galactosidase was observed.

Effect of carbon sources

Effect of different carbon sources was studied to know the best carbon source for the production of β galactosidase by isolate MH2. Different sugars such as glucose, lactose, sucrose, starch, xylose, frustose, dextrose and inositol were supplemented in the production medium and β galactosidase activity was measured. Among the sugars, glucose, lactose, sucrose, xylose and dextrose enhanced the production of β galactosidase as compared to the unsupplement (without carbon source). As compared to control (medium 2), medium containing xylose showed highest activity of 457.55 U/mg (Figure 3) whereas starch, fructose and inositol decreased the activity of the isolate MH2. El-Kader *et al* [22] studied different carbon sources such as lactose, sucrose, glucose, fructose, starch, galactose, dextrane and inositol for enzymatic production by *Bacillus* isolate No. 12. Carbon concentration for each carbon sources was used as that of basal medium (10g/l). The results indicated that lactose supported better yields of enzymatic activity of 390 U/ml as compared to other carbon sources whereas glucose gave the lowest activity of 100 U/ml and productivity of the test enzyme.This result was almost similar with Hsu *et al* [23] who found that the final viable population of *B. longum* CCRC 15708 was higher in cultures containing either lactose as the sole carbon source with the highest β galactosidase activity detected with lactose followed by galactose and the lowest activity with glucose as the carbon source.

Effect of nitrogen sources

Effect of different nitrogen sources was studied to know the best nitrogen source for the production of β galactosidase by MH2 isolate. Yeast extract, beef extract, peptone, casein, urea and ammonium sulphate were supplemented in the production medium and β galactosidase activity was measured. Yeast extract, beef extract and peptone enhanced the production of β galactosidase as compared to the unsupplemented medium. Medium containing yeast extract showed highest activity of 366.53 U/mg (Figure 4) whereas casein, urea and ammonium sulphate decreased the activity of β galactosidase. Kumar *et al* [24] reported maximum β galactosidase activity among various nitrogen sources like peptone, beef extract, sodium carbonate, ammonium sulphate and ammonium chloride at 1 %

(w/v) concentration. Peptone was found to be the better nitrogen sources favoring maximum enzyme production.

Effect of metal salts on β galactosidase enzyme

In order to investigate the effect of metal salts as cofactor for β galactosidase activity, metal salts were individually supplemented in the β galactosidase assay medium. Effect of different metal salts such as $CaCl_2$, $CuSO_4$, $FeSO_4$, $MgCl_2$, NaCl and $ZnSO_4$ wasstudied with concentration ranging from 0 to 10 mM. β galactosidase activity of isolate MH2 is presented in graphical form as shown below (Figure 5). The β galactosidase activity was inhibited at 10 mM concentration of Fe^{2+}, Mg^{2+} and Zn^{2+} respectively, on the other hand β galactosidase activity was enhanced in the presence of Ca^{2+}, Cu^{2+} and Na^{+}. Overall, $FeSO_4$, $MgCl_2$ and $ZnSO_4$ showed more enzyme inhibition as compared to $CaCl_2$, $CuSO_4$ and NaCl metal salts at 10 mM concentration. Somyos Osiriphun and Phimchanok Jaturapiree [25] studied the effects of various cations on the activity of β galactosidase from B1.2, each in final concentrations of 1, 10 and 100 mM and found that, the addition of monovalent cations Na^{+} and K^{+} had no affect on enzyme activity. The highest activities of B1.2 β galactosidases were observed in the presence of 1 mM Fe^{2+} and 10 mM Mg^{2+}, similarly, of 1-10 mM Mn^{2+} and Co^{2+}. The presence of 1 and 10 mM Cu^{2+} decreased B1.2 activity by 40 % and the activity of B1.2 β galactosidase was also completely inhibited by the addition of 10 mM Fe^{2+}. The inhibitory effects of 10 mM Zn^{2+} and Cu^{2+} was similar in that the enzyme activity was reduced by 30%. All divalent cations completely inhibited β galactosidase activity at a final concentration of 100 mM. A similar result was reported for other thermophilic strains of *Talalomyces thermophilus* CBS 236.58 [26].

Comparative study of β galactosidase activity in immobilized state and free state of enzyme

Thermal stability

Incubation of the strain MH2 at different temperatures revealed that the optimum temperature was found to be 37° C (Figure 6) for free enzyme and 40° C for immobilized enzyme under same conditions. Both free and immobilized enzymes were stable upto 50° C and a remarkable drop at 70° C was observed for the both immobilized and free enzyme from *Escherichia coli* MH2. Ansari and Husain [27] recorded the activity profile of soluble and immobilized β galactosidase. The activity of soluble and immobilized β galactosidase was assayed in 0.1 M sodium

acetate buffer, pH 5.5 at various temperatures (20-80° C) for 15 min. Results showed that, the temperature optimum for both the soluble and immobilized enzyme was same, i.e. 50° C. Immobilized β galactosidase exhibited greater fractions of enzyme activity at lower and higher temperatures as compared to its soluble counterpart. The soluble enzyme showed 41 % activity at 30° C whereas the immobilized β galactosidase retained 37 % activity at the same temperature.

pH stability

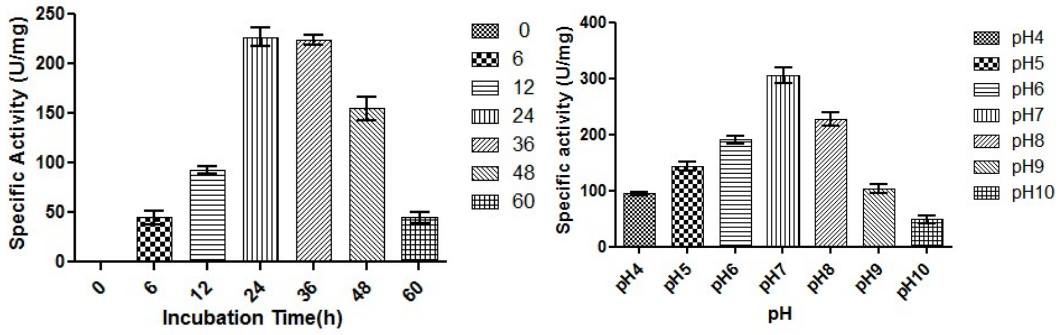
The pH effect was studied on the activity of free and immobilized β galactosidase (Figure 7). pH values ranged from 4.0 to 10pH. For bacterial isolate, *Escherichia coli* MH2, the pH optima for both free and immobilized β galactosidase was 7.0, after that a remarkable drop at pH 9.0 was observed for the both immobilized and free enzyme. Ansari and Husain [27] observed the activity profile of soluble and immobilized β galactosidase. The activity of soluble and immobilized β galactosidase was measured in the buffers of various pH (2.0-9.0). The pH optimum for both soluble and immobilized enzyme was same at 5.5 and immobilized β galactosidase exhibited a remarkable broadening in pH activity profiles as compared to the native enzyme. Results showed that the soluble enzyme retained 40 % activity at pH 4.0 while the immobilized β galactosidase exhibited 88 % enzyme activity at the same pH.

Incubation Time

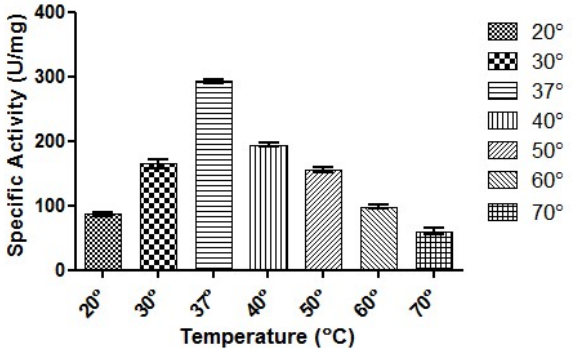
Effect of reaction time of immobilized and free β galactosidase enzyme was studied (Figure 8). Activity of β galactosidase enzyme was determined at different incubation time intervals 5, 10, 15, 20, 25 and 30 min for enzyme assay. Free β galactosidase showed maximum activity at 25 min of incubation, whereas immobilized β galactosidase showed maximum activity in 15 min of incubation.

Storage stability

For the free β galactosidase, enzyme activity declined after storage of 15 days at 4° C for *Escherichia coli* MH2. In case of immobilized β galactosidase, enzyme activity was stable upto 25 days and after that started declining (Figure 9). This signifies that coupling of immobilized enzyme improved the stability of β galactosidase. Similarly, Dwevedi and Kayastha [28] studied stability of free and immobilized enzyme for a period of 50 days at 4° C and the residual activity was checked from time to time. Results showed that, there was no loss in activity of immobilized β galactosidase till 40 days whereas free enzyme was stable for only 10 days at 4° C.



(a) (b)



(c)

Figure 1: The effect of incubation time, pH and temperature on β galactosidase production

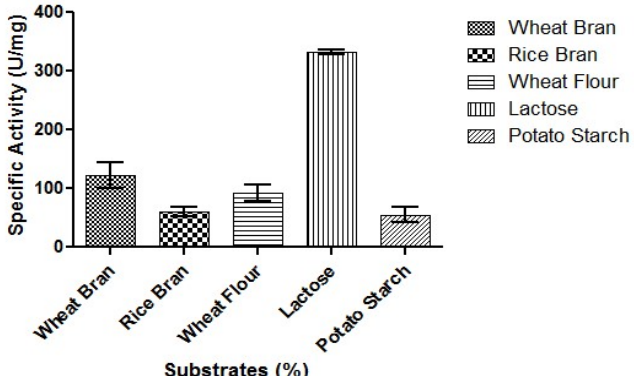


Figure 2: Effect of different substrates on β galactosidase production

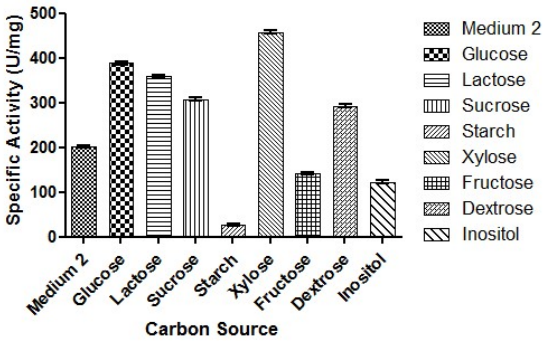


Figure 3: Effect of various carbon sources on β galactosidase production

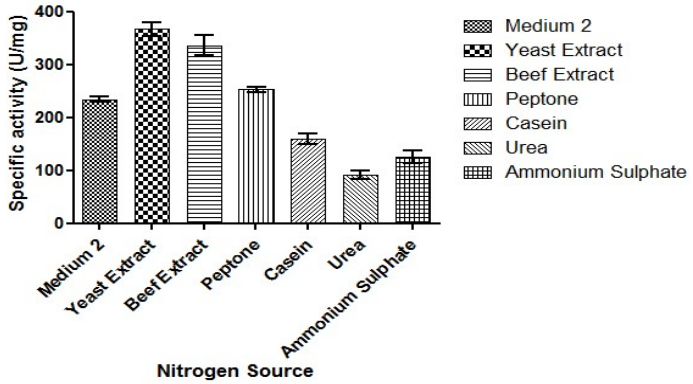
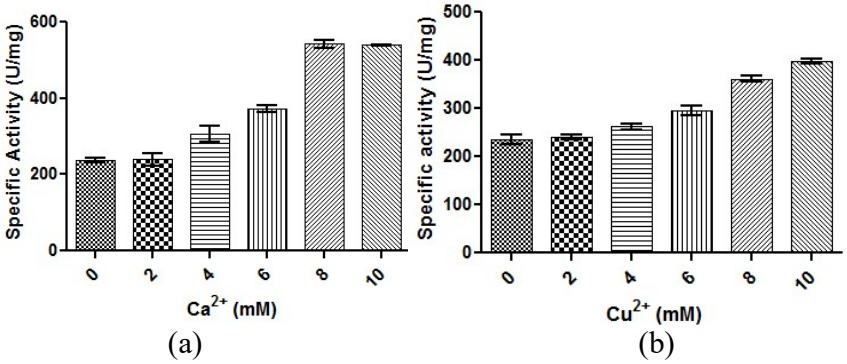
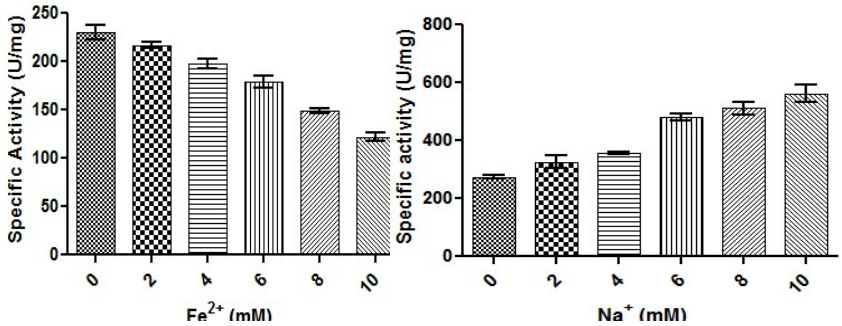
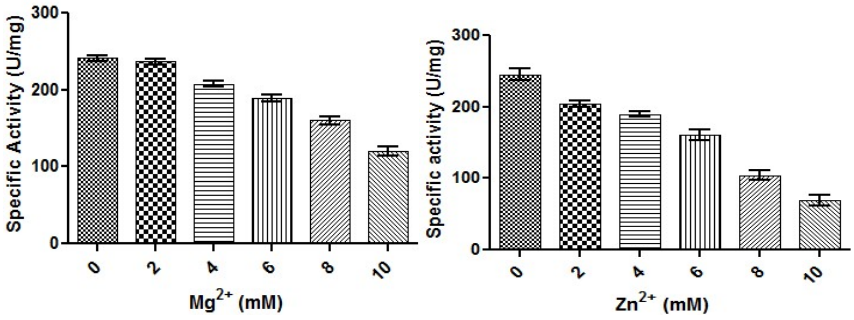


Figure 4: Effect of various nitrogen sources on β galactosidase production.

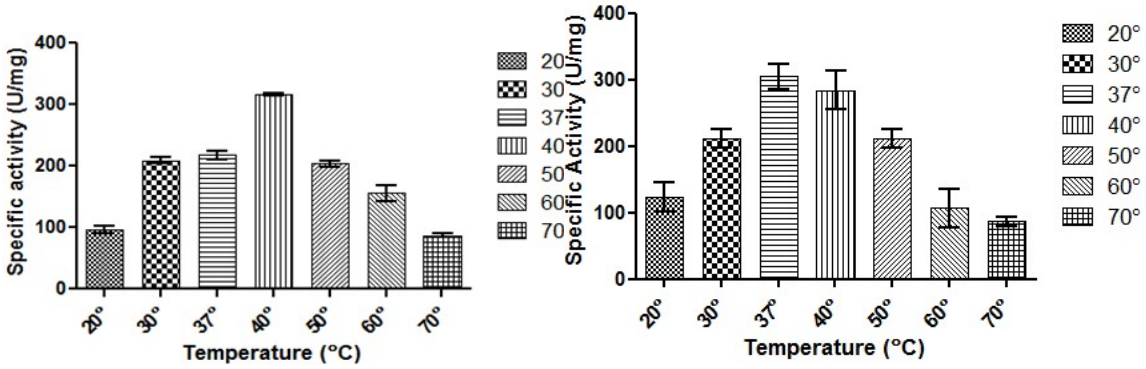






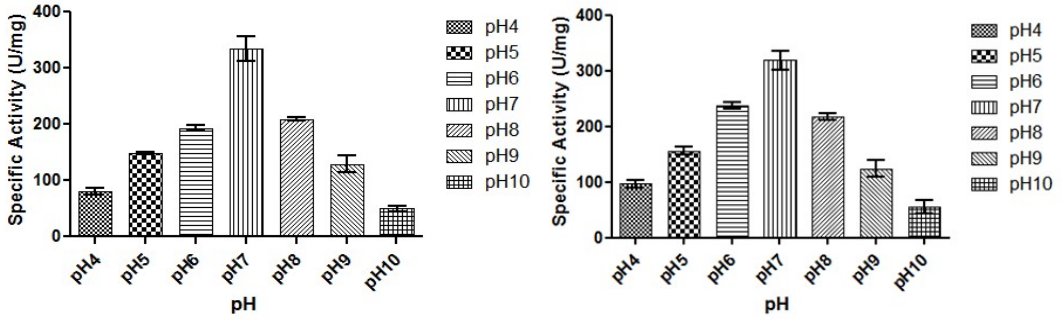
(e) (f)

Figure 5: Effect of metal ions on β galactosidase production.



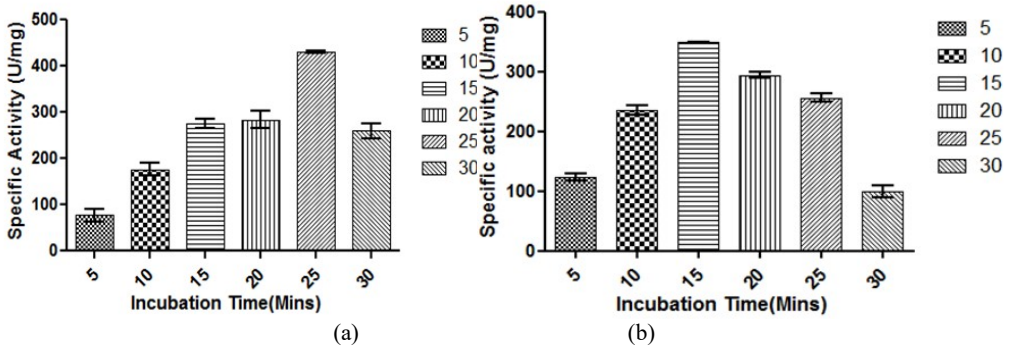
(a) (b)

Figure 6: Plot of the specific activity *vs.* temperature to determine the temperature stability of β galactosidase.



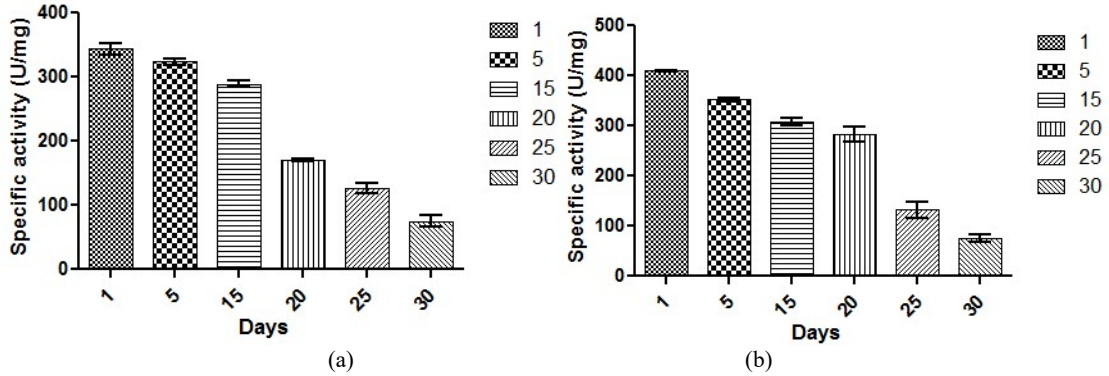
(a) (b)

Figure 7: Plot of the specific activity *vs.* pH to determine the pH stability of β galactosidase.



(a) (b)

Figure 8: Plot of the specific activity *vs.* incubation time to determine the incubation time stability of β galactosidase.



(a) (b)

Figure 9:Plot of the specific activity *vs.* storage stability (days) to determine the storage stability of β galactosidase

CONCLUSION

From the above results, it can be concluded that *Escherichia coli* MH2 can efficiently produce β galactosidase. The optimum pH and temperature were found to be 7.0 and 37° C respectively for β galactosidase production by *Escherichia coli* MH2. Xylose and yeast extract was found to be best carbon and nitrogen source. Immobilized enzyme was significantly more stable against free enzyme as there was no loss in activity of immobilized β galactosidase till 25 days whereas enzyme activity of free enzyme was stable for only 15 days. This suggests that *Escherichia coli* MH2 can be a potential producer of β galactosidase which could find applications in industry and biotechnology.

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