PRODUCTION OF LACTASE FROM MICROORGANISM AND PURIFICATION OF IT

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Abstract:

Enzymes bind with chemical reactants called substrates. There may be one or more substrates for each type of enzyme, depending on the particular chemical reaction. This research is based on this enzyme-substrate interaction for the microorganism. For the specific substrate, the organisms will secrete the specific enzyme to digest that substrate. Lactose as a substrate is taken for the production of lactase by the *E.coli*. In this research, production of lactase (*in situ*)from the *E.Coli* is optimized. Temperature, substrate concentration, time and pH are taken as the production parameters for the lactase production. The isolation of the lactase is carried out by the aqueous two phase system (ATPS). PEG 4000, anhydrous potassium phosphate and water is used for the preparation of ATPS. Lactase enzyme activity was measured by the rate of hydrolysis of *o*-nitrophenyl β-D-pyranogalactoside.The efforts to tackle these diseases like lactase and sucrase-isomaltase deficiency, glucose-galactose malabsorption, galactosaemin, hereditary fructose intolerance and the glycogen storage diseases will be further strengthened.

Keywords: lactase, β-galactosidase, *E.coli*, aqueous two phase system.

Introduction

It is being observed that, there is continuous development in the areas of biotechnology since 1850. The area of biotechnology involves various fields like molecular biology, fermentation, genetic engineering, and downstream processing of biomolecules. Biomolecules include proteins, enzymes, pigments etc. amongst these; enzymes are gaining a lot of importance today because of their varied functions and occurrence. The β-galactosidase from E. coli was instrumental in development of the operon model, and today is one of the most commonly used enzyme in molecular biology.

β-Galactosidase (Lactase EC. 3.2.1.23) catalyzes the hydrolysis and transgalactosylation reaction of β-D-galactopyranosides, such as lactose. The enzyme occurs in a wide variety of organism including microorganisms, plants and animals. The application ofβ-galaclosidase in bioprocess technology has been achieved exclusively with microbial enzymes, which have long been used for the hydrolysis of lactose for increasing the digestibility of milk or for improving the functional properties of dairy products. During the past decade, another potential application ofthe enzyme has also been developed; the β-galactosidase catalyzed transgalactosylation has proved to be useful for the structural and functional modifications of food materials, medicines, and other biologically active compounds.

*E. coli*lacZ β-galactosidase is the best characterized β-galactnsidase; its three dimensional structure was already known (Jacobson et al., I994). The regulation mechanism of the transcription of the lacZ β-galactnsidase gene was established as the operon model. The extensive structural and mechanistic studies of the E. coli lacZenzyme have provided the basis for the detailed insights into β-galactnsidase catalysis.

However, the usefulness of this enzyme in bioprocees technology has been examined only on the laboratory scale; *E.coli* lacZ enzyme stops short of making a contribution in the food industry mainly

because of the unacceptability of the bacterium in the uses related to food. Rather, the *E.coli*lacZ β-galactnsidase and its gene are essential tools in genetic engineering, immunochemical, and molecular biology studies.

The *E. coli* lacZ β-galactnsidase is a tetrameric protein consisting of identical subunits of an 1173 amino acids polypeptide chain, which folds into five sequential domains with an extended segment at the amino terminus (Jacobson et al., 1994). The enzyme has four active sites per tetramer, each of which is made of elements from two different subunits. All the catalytically important amino acid residues are located in the domain three of the subunit. The enzyme requires the essential divalent and monovalent cations for its maximum activity, but the role ofthese metal activators in the enzyme mechanism is not yet known in detail. The proposed mechanisms of the hydrolysis and transgalactosylation reactions catalyzed by the *E.coli* lacZβ-galactosidase are reviewed. The "α - complementation" phenomenon would be worth nothing as a unique characteristic of this enzyme. A fragment of amino terminal peptide, which consists of approximately 50 amino acids encoded by a plasmid, is capable of intraallelic α-complementation with a defective form of the β-galactosidase encoded by the host. The α-complementation has been used for designing plasmid vectors that permit histochemical identification of recombinant clones in genetic engineering studies.

Lactose (4-*O*-β-*D*-galactopyranosyl-*D*-glucopyranose) is the principal carbohydrate of milk, occurring at concentration of about 5% w/v. It is hydrolyzed to equimolar concentrations of glucose and galactose by enzymes termed,β-galactosidase, commonly referred to as lactases. Since cow's milk and various products made from it are major food items, lactose constitutes a significant portion of the daily carbohydrate intake of human beings.

Downstream processes reported for the purification ofβ-galactosidase include acetone precipitation, ammonium sulphate precipitation, chromatographic methods like gel filtration, ion-exchange, hydrophobic interaction, and aqueous two phase system, a maximum purification of around 1000 fold is reported to have been achieved by crystallization (Mayazaki., 1988).

Major advantage has occurred in recent years in the development of technique for the separation of peptides and proteins. With the availability of many proteins through the advent of genetic engineering technique, the criteria for establishing biorecovery, purification strategies for proteins purity are currently undergoing substantial reexamination. Central to these advances are the requirement to develop the new strategies for the purification of specific peptide or proteins from complex mixtures. The very high purity of proteins can be achieved only by the application of several high-resolution separations while maintaining its bioactive form. One such purification technique involves partitioning of biomolecules between two immiscible aqueous phase i.e. aqueous two-phase systems (ATPS). ATPS have high water content and hence provide a protective and gentle environment for the partitioning of the biomolecules (Albenssan et al, I987). Thus rule out the harsh treatment offered by traditional extraction systems. ATPS are ideal technique when: clarification, concentration, and purification can be integrated in one step (Gupta et al., 1999). Partitioning of the biopolymer between the phases is dependent on the number of factors, including types of polymer composing two phase, average molecular weight of the polymer, molecular distribution of polymer, ionic strength, pH, and temperature (Kula at el., I982). This partitioning of proteins can be significantly and selectively influenced by covalently binding various chemical groups to the phase forming polymers (Gote at el., I984).

In this present work, the isolation of the intracellular enzyme β-galactosidase from *E. coli* by separation in aqueous two-phase systems was studied with the purpose to obtain a simple and inexpensive separation procedure.

The hereditary disorders of carbohydrates metabolism include the congenital disaccharides deficiencies, especially lactase and sucrase-isomaltase deficiency, glucose-galactose malabsorption, galactosaemin, hereditary fructose intolerance and the glycogen storage diseases can be address through this research.

Materials and Methods for Optimization:

Strain: The strain of *E. coli* K-12 MTCC I302used for the present work was procured from Institute of Microbial Technology, Chandigarh. It was maintained by monthly sub-culturing in media mention below.

Media: It was prepared by adding 1.0% NaCl,1.0% Tryptone and 0.5% Yeast Extract in Distilled Water. These media chemicals were obtained from Himedia Ltd, India.

Inoculum Preparation: 18 hr old culture slants were used for preparing lnoculum. These slants culture cells were suspended in distilled water such that the absorbance should be 0.56 at 610 nm. 1 ml of this inoculum was added to the 50 ml (i.e. 2% v/v) of production media. Lactose was used as a substrate for inducing lactase synthesis. This procedure was kept constant for optimization ofall production parameters ofenzyme.

Standard Enzyme: β-Galactosidase (Lactase) was obtained from Sigma-Aldirich Chemicals, USA. It was stored in refrigerator at– 4.0 -0^{0}C for retaining its activity. ONPG substrate was obtained from Himedia Ltd, India. Chemicals required for optimization and purification were of analytical grade and were purchased from S. D. Fine Chemicals and Merck, India.

Reagents and Solutions:

1. **Magnesium Solution:** 2.465 g of magnesium sulphate heptahydrate [$MgSO_4.7H_20$] was dissolved in about 95 ml of water. This solution was transferred into a 100 ml volumetric flask; volume was made up with water and mixed.
2. **EDTA Solution:** 0.186 g of disodium EDTA dihydrate [$C_{10}H_{14}N_2Na_2O_8.2H_20$] was dissolved in about 95ml of water. This solution was transferred into a 100 ml volumetric flask; volume was made up with water and mixed.
3. **P.E.M Buffer:** 0.88 g of potassium dihydrogen phosphate [KH_2PO_4] and 0.8 g of dipotassium hydrogen phosphate trihydrate [$K_2HPO_4.3H_2O$] were dissolved in about 90 ml of water. 1 ml of magnesium solution and 1 ml of EDTA solution were added to it. This solution was transferred into a 100 ml volumetric flask, and volume was made up with water and mixed. The pH was adjusted 7.2 --7.3.
4. **ONPG Substrate:**4 mg of ONPG was dissolved into 1 ml of buffer. 25 ml of such solution was prepared and mixed.
5. **Sodium Carbonate Solution:** 5 g of sodium carbonate anhydrous [Na_2CO_3] and 3.72 g of disodium EDTA dihydrate were dissolved in about 90 ml of water. This solution was transferred into a 100 ml of volumetric flask and volume was made up with water and mixed
6. **Standard*o*-Nitrophenol Solution:** 13.9 mg of *o*-Nitrophenol was dissolved into 1 ml of 96% ethanol in 100 ml volumetric mask; and volume was made up with water and mixed.

Preparation of Standard Curve:

2, 4, 6, 8,10, 12, &14 ml of standard *o*- Nitrophenol solution were pipetted into 100 mL of volumetric flasks. 25 ml of sodium carbonate solution was added to each. Each was diluted to volume with P.E.M buffer and mixed. The dilutions were contained respectively 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.14 μ/mol of

o-Nitrophenol. The absorbance of each dilution was observed at 420 nm in 3 ml cuvette cell,with suitable UV-1650PC Visible Spectrophotometer (SHIMADZU, Japan) using water as a blank. For each solution, absorbance was plotted against μ/mol of *o*- Nitrophenol. The extinctioncoefficient at these dilutions was obtained by calculating the slope ofline i.e. Y.

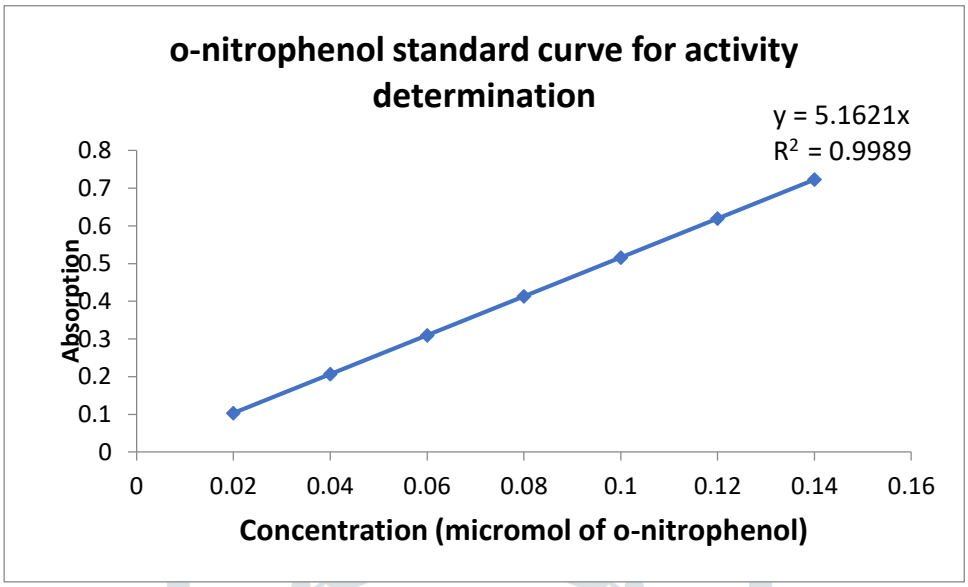

Figure 1: standard curve

Assay Method:

- 0.25 ml of enzyme sample + 1.25 ml of ONPG solution were taken.
- It was kept at 37^0C for 10 min incubation.
- 0.5 ml of1M Na_2CO_3 was added.
- The absorbance at 420 nm was observed within 20 min.

Blank Solution:

- 0.5 ml of 1 M Na_2CO_3 was taken.
- It was kept at 37 ^{0}C for 10 min incubation.
- 1.25 ml of ONPG solution was added.
- The absorbance at 420 nm was observed just after adding 0.25 ml of enzyme-solution.

Enzyme activity: Enzyme activity was measured by the rate of hydrolysis of *o*-nitrophenyl β-D-pyranogalactoside (ONPG). The enzyme activity was determined by the rate of increase of optical density at 420 nm (owing to the liberation of *o*-nitrophenol) as measured in the UV-1650PC Visible Spectrophotometer (SHIMADZU, Japan) at room temperature & at pH 7.0. Enzyme activity was expressed in units/ml. One β-galactosidase unit is defined as the quantity of enzyme that will liberate 1 μg *o*-nitrophenol per minute per ml of enzyme solution under the condition of assay.

The activity ofenzyme:

$$\textbf{Activity} = [Y \times 139 \times D.F.] / [5.1621 \times 10 \times 0.25]$$

Activity - Activity expressed units/ml
Y - The average of the absorbance readings for the sample, corrected for the sample blank
D.F. - Total dilution factor of the sample
10 - Incubation time in minute
0.25 - Enzyme sample taken for assay in ml

Optimization of Production Parameters

A] Optimization of the Time:

- 50 ml media was prepared and were autoclaved for 15 min at 15 Kg/cm^2.
- 2 % inoculum was added to it and fermentation was started in Incubator Shaker at 200 rpm & at 37°C.
- 2 ml samples were taken out at 0 hr, 2hr, 4hr, 8 hr, 10 hr, 12 hr, 15 hr,17 hr, 18 hr, 20 hr, 22 hr and 24 hr.

Cells were Centrifuged at 5,000 rpm for 10 min and washed with saline and then sonicated. Cell debris was removed by centrifugation. The enzyme solutions were collected. Assay method was followed to determine the activity of enzyme samples.

Every experiment was performed in duplicate and two samples were taken from each batch. As such there were four readings and average of it was considered as a desired reading.

B]Optimization of the Temperature:

- 50 ml media was prepared and were autoclaved for 15 min at 15 Kg/cm^2.
- 2 % inoculum was added to it and fermentation was started in Incubator Shaker at 200 rpm.
- Cells were incubated at various fermentation temperatures as 28 °C, 31 °C, 34 °C, 37 °C, 40 °C, 43 °C and 46 °C up to 18 hours.

Cells were centrifuged at 5,000 rpm for 10 min & washed with saline and then sonicated. Cell debris was removed by centrifugation. The enzyme solutions were collected. Assay method was followed to determine the activity of enzyme samples.

C] pH of the Media:

- The media at different initial pH (6.0, 6.3, 6.6, 6.9, 7.2, 7.5, 7.8 and 8.1) was prepared and were autoclaved for 15 min at l5 Kg/cm^2.
- 2 % inoculum was added to it and fermentation was started in Incubator Shaker at 200 rpm.
- The cells were incubated in the media for 18 hours and at 37 °C.

Cells were Centrifuged at 5,000 rpm for 10 min & washed with saline and then sonicated. Cell debris was removed by centrifugation. The enzyme solutions were collected. Assay method was followed to determine the activity of enzyme samples.

D] Concentration of the Substrate:

- 50 ml media was prepared.
- To this media, lactose at various concentrations was added and media was autoclaved for 15 min at 15 Kg/cm^2.
- The cells were incubated in media for 18 hours, 37 °C, and pH 7.2.

Cells were centrifuged at 5,000 rpm for 10 min & washed with saline and then sonicated. Cell debris was removed by centrifugation. The enzyme solutions were collected. Assay method was followed to determine the activity of enzyme samples.

Materials and Methods for the Purification:

For the isolation of an enzyme from the disintegrated cell suspension of E. coli, ATPS was suitable method. In PEG - Salts system, an enzyme was acquired in the PEG rich phase specifically, leaving all other DNA, RNA, liposome in the bottom salt phase. More than 95 % of the total enzyme could be isolated into the PEG top phase. The systems were studied at different concentration of PEG and salts to find out the maximum recovery of the enzyme in top PEG phase.

Polyethylene glycol (PEG) 4000, MW = 3600-4400, and salt Sodium Sulphate were obtained from S.D. Fine Chemicals, India. All materials used were of laboratory reagent grade.

Preparation of the aqueous two-phase system:

System: PEG 4000 + Potassium phosphate (anhydrous) + Water
Temperature: Room temperature

- Appropriate amounts of each compound were taken.
- Distilled water was added to each system.
- Total weight of each system was kept at 3. 4 gm.
- To these systems,1 gm of disintegrated cells suspension (50 % w/w) was added.
- The system was allowed to form two phases. Most of the enzyme was extracted in the upper PEG rich phase while the cell debris, DNA, and RNA was at bottom phase.
- The volume of both phase were measured.
- For the measurement of the protein concentrations and activities in both phases Bradford and ONPG assay method was used respectively.

Standard Curve for the Protein Concentration Measured:

Preparation of the Protein Reagent: Coomassie Brilliant Blue G - 250 (100 mg) was dissolved in 50 ml 95% ethanol. To this solution 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter. Final concentrations in the reagent were 0.01% (w/v), CBB G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid.

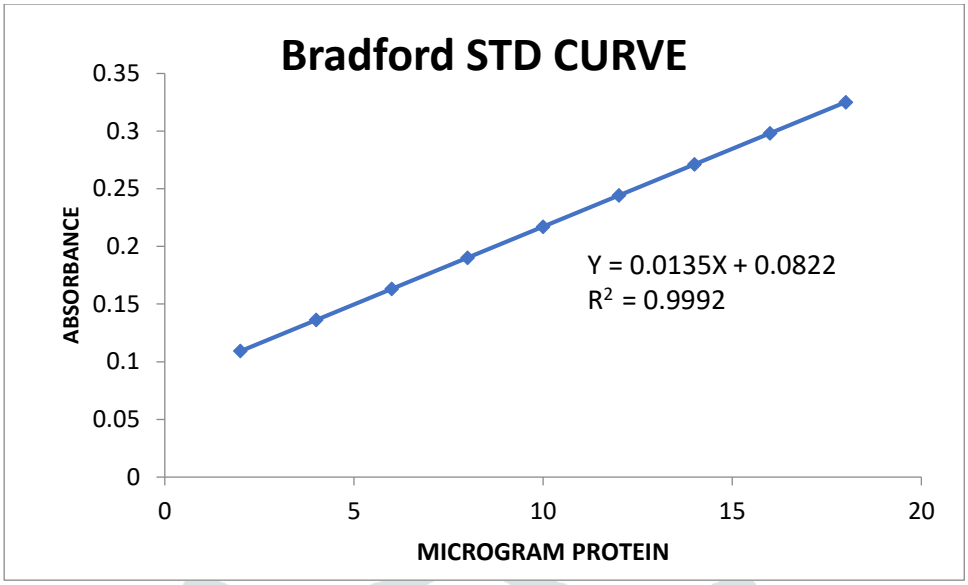

Figure 2: Bradford std. curve for determination of the protein concentration

Micro Protein Assay: Protein solution containing 2 to 20 µg protein in 0.2 ml phosphate buffer was taken into small test tubes. 2 ml of protein reagent was added to the test tubes and the contents mixed. Absorbance at 595 nm was observed in 3 ml cuvettes against the reagent blank prepared from 0.2 ml of phosphate buffer and 2 ml protein reagent.

Result and Discussing

β-Galactosidase Induction:

The formation of an enzyme in response to an inducing substance has been termed enzymatic adaptation. Adaptation by microorganisms to the utilization of certain nutrients has been considered to represent the *de novo* formation of an enzyme as a specific response to the presence of its specific substrate. The lac operon is the classic example of gene regulation, in which the production of β-galactosidase (lactase) is induced by the presence of lactose in growth medium.

Optimization of the Time:

Table 1 shows the absorbance reading of each enzyme sample and their corresponding dilution factor. Table 2 shows an activity of each enzyme sample taken at the different time intervals. There was 0 Units/ml activity at the initial time of 0 minute. As the time of growth was increased the production of enzyme also increased. This was because of the increment in the cell mass with enzymatic adaptation. The maximum production of β-galactosidase was seen at the growth of 18 hours. Thus the fermentation time for the maximum production of an enzyme was 18 hours. The activity was getting lowered after the fermentation time of l8 hr. Product inhibition was one of the factor that inhibit enzyme activity and therefore enzyme solution showed lesser activity. The figure 3 shows that at 18 hr it has peak and corresponding activity was found to be 25.76 Units/ml.

Table 1: Absorbance of the enzyme solutions at the Different Fermentation Time

Time in Hr	Absorbance Y1	Absorbance Y2	Absorbance Y3	Absorbance Y4	D.F.
0	0	0	0	0	0
2	0.002	0.002	0.015	0.002	1
4	0.081	0.098	0.098	0.100	1
8	0.125	0.169	0.133	0.145	1
10	0.151	0.191	0.183	0.200	1
12	0.187	0.233	0.213	0.225	4
15	0.284	0.338	0.324	0.277	4
17	0.359	0.452	0.358	0.319	4
18	0.642	0.602	0.523	0.625	4
20	0.598	0.502	0.513	0.531	4
22	0.578	0.464	0.473	0.493	4
24	0.564	0.464	0.457	0.471	4

Table 2: Activities of the enzyme solutions at the different fermentation time

Activity1 Units/ml	Activity2 Units/ml	Activity3 Units/ml	Activity4 Units/ml	Average Activity
0	0	0	0	0
0.016	0.015	0.025	0.025	0.02 ± 0.011
0.876	1.051	1.050	1.077	1.014 ± 0.185
1.348	1.814	1.431	1.561	1.539 ± 0.407
1.630	2.062	1.971	2.157	1.955 ± 0.698
8.071	10.033	9.198	9.684	9.246 ± 0.666
12.266	14.544	13.943	11.947	13.175 ± 0.632
15.463	19.482	15.427	13.722	16.023 ± 0.223
27.638	25.952	22.538	26.911	25.759 ± 1.128
25.772	21.612	22.114	22.879	23.095 ± 0.930
24.902	20.007	20.383	21.240	21.633 ± 1.119
24.308	20.007	19.699	20.292	21.076 ± 1.204

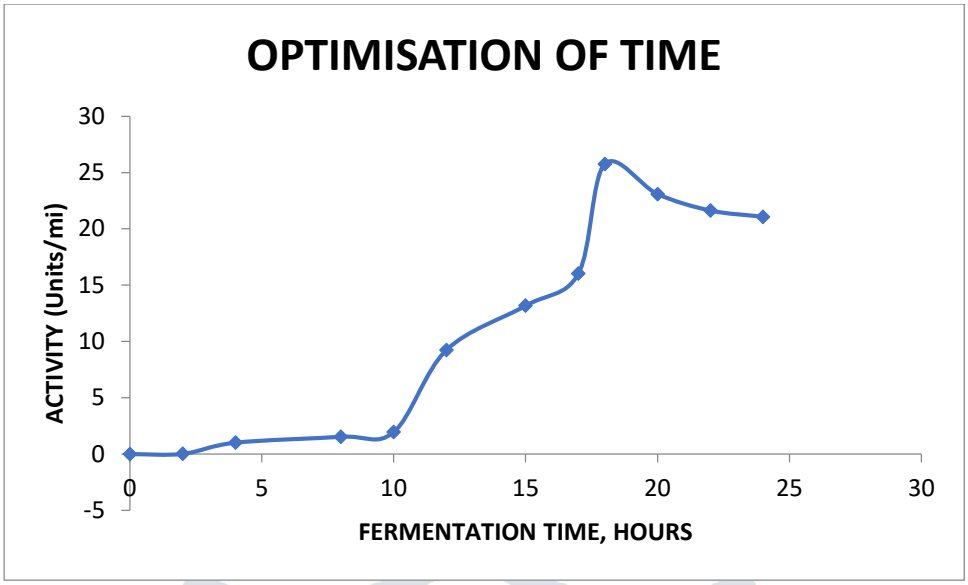

Figure 3: The activity of β-galactosidaseas a function of the Fermentation Time

Optimization of the temperature:

Table 3 shows the absorbance reading of each enzyme sample and their corresponding dilution factor. Table 4 shows an activity of each enzyme sample taken at the different temperatures. The fermentation was carried out at various temperatures. The maximum production of an enzyme was at the fermentation temperature of 37°C. At 28°C there was production of an enzyme, but that was less compare to that of 37°C. As fermentation was carried out at higher temperatures, the production of an enzyme diminished. Figure 4 also shows the peak at 37°C corresponding to the activity of 20.86 Units/ml. Thus it is concluded that the optimum fermentation temperature for the production of an enzyme was 37°C.

Table 3: Absorbance of the enzyme solutions at the different fermentation temperatures

Temperature in ^{0}C	Absorbance Y1	Absorbance Y2	Absorbance Y3	Absorbance Y4	D.F.
28	0.347	0.340	0.337	0.339	3
31	0.421	0.383	0.432	0.446	3
34	0.533	0.518	0.585	0.501	3
37	0.503	0.493	0.498	0.443	4
40	0.593	0.570	0.503	0.493	3
43	0.285	0.258	0.305	0.318	3
46	0.405	0.355	0.403	0.418	1

Table 4: Activities of the enzyme solutions at the different fermentation temperature

Activity1 Units/ml	Activity2 Units/ml	Activity3 Units/ml	Activity4 Units/ml	Average Activity
11.21	10.99	10.89	10.95	11.01 ± 0.07
13.62	12.39	13.98	14.41	13.60 ± 0.32
17.22	16.74	18.90	16.19	17.26 ± 0.58
21.67	21.24	21.46	19.09	20.86 ± 0.59
19.16	18.42	16.25	15.93	17.44 ± 0.79
9.21	8.34	9.86	10.28	9.42 ± 0.42
4.36	3.82	4.34	4.50	4.23 ± 0.14

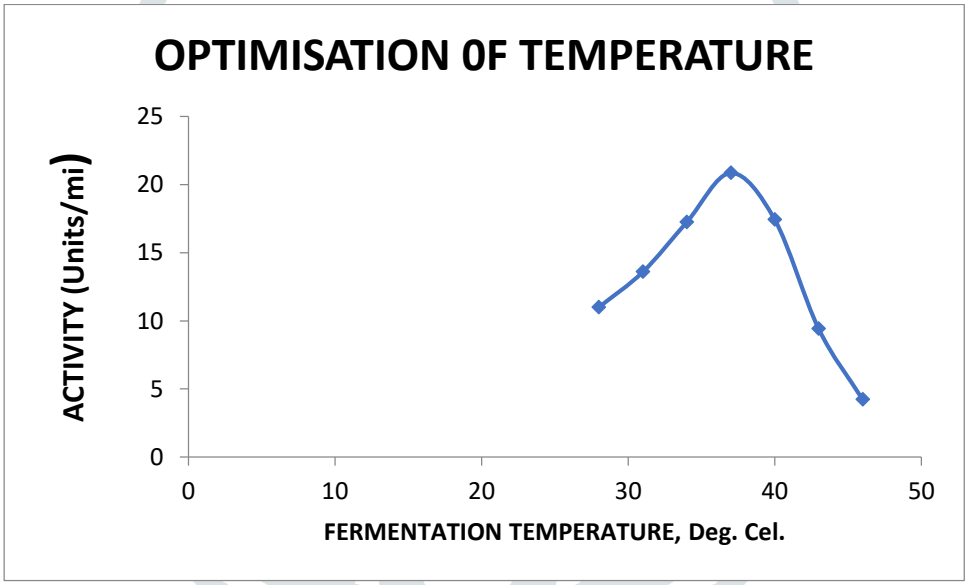

Figure 4: The activity of β-galactosidase as a function of the Fermentation Temperature

Optimization of pH of the Medium:

The fermentation was carried out at various pH from 6.0 to 8.1. Table 5 shows the absorbance reading of each enzyme sample and their corresponding dilution factor. Table 6 shows an activity of each enzyme sample taken at the different pH. It was shown that the fermentation at pH 7.2 gives the maximum production of an enzyme. The activity was 26.54 Units/ml at pH 7.2. Toward the acidic pH range below pH 7.2 the *E.coli* was not prone to induce an enzyme. The same case was found toward the basic pH range above pH 7.2. Figure 5 also shows the peak at pH 7.2 and its corresponding activity was 26.54Units/ml. Thus the maximum production of an enzyme was found to be at pH 7.2 and it could be considered as optimum pH.

Table 5: Absorbance of the enzyme solutions at the different fermentation pH

pH	Absorbance Y1	Absorbance Y2	Absorbance Y3	Absorbance Y4	D.F.
6.0	0.1351	0.1096	0.1703	0.1234	1
6.3	0.3097	0.223	0.2788	0.2316	1
6.6	0.6411	0.5975	0.6203	0.6014	1
6.9	0.7718	0.7914	0.8132	0.8016	2
7.2	0.6052	0.6314	0.5889	0.6391	4
7.5	0.573	0.6093	0.4561	0.5055	3
7.8	0.4039	0.3986	0.3541	0.322	1
8.1	0.2691	0.2938	0.3225	0.2645	1

Table 6: Activities of the enzyme solutions at the different fermentation pH

Activity1 Units/ml	Activity2 Units/ml	Activity3 Units/ml	Activity4 Units/ml	Average Activity
1.45	1.18	1.83	1.33	1.45 ± 0.14
3.33	2.40	3.00	2.49	2.81 ± 0.22
6.91	6.44	6.68	6.48	6.62 ± 0.11
16.63	17.05	17.52	17.27	17.11 ± 0.19
26.08	27.20	25.37	27.53	26.54 ± 0.50
18.52	19.69	14.74	16.33	17.32 ± 1.11
4.35	4.29	3.814	3.47	3.98 ± 0.21
2.89	3.16	3.47	2.85	3.096 ± 0.14

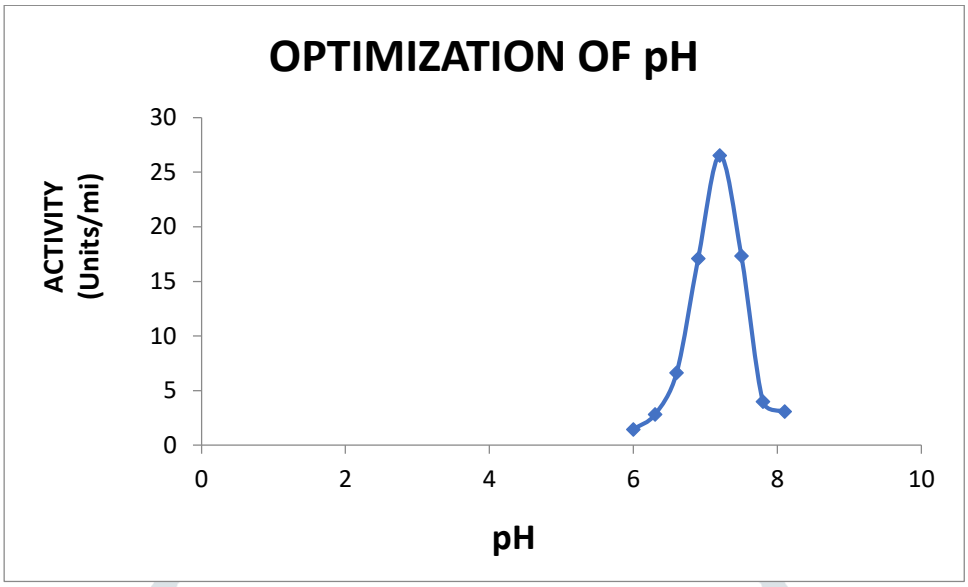

Figure 5: The activity of β-galactosidaseas a function of Initial pH of the Medium

Optimization of Substrate Concentration:

The fermentation was carried out at various lactose concentrations from 0.4 to 4.4 (w/w). Table 7 shows the absorbance reading of each enzyme sample and their corresponding dilution factor. Table 8 shows an activity of each enzyme sample taken at the different lactose concentrations. It was shown that the fermentation at 1.0% lactose concentration gives the maximum production of an enzyme. The activity was 19.12 Units/ml at 1.0 % lactose concentration. Figure 6 also shows the peak at 1.0% lactose concentration and its corresponding activity was 19.12 Unit/ml. Thus, the maximum production of an enzyme was found to be at 1.0% lactose concentration and it could be considered as optimum lactose concentration.

Table 7: Absorbance of the enzyme solutions at the different % lactose concentration (w/w)

% Lactose Concentration (w/w)	Absorbance Y1	Absorbance Y2	Absorbance Y3	Absorbance Y4	D.F.
0.40	0.342	0.409	0.345	0.332	4
1.00	0.451	0.428	0.436	0.46	4
2.00	0.364	0.448	0.388	0.398	4
2.80	0.331	0.372	0.352	0.362	4
3.60	0.330	0.374	0.349	0.341	4
4.40	0.322	0.373	0.274	0.308	4

Table 8: Activities of the enzyme solutions at the lactose concentration (w/w)

Activity1 Units/ml	Activity2 Units/ml	Activity3 Units/ml	Activity4 Units/ml	Average Activity
14.73	17.62	14.87	14.30	15.39 ± 0.76
19.43	18.44	18.78	19.82	19.12 ± 0.31
15.68	19.30	16.72	17.15	17.21 ± 0.76
14.26	16.03	15.17	15.59	15.26 ± 0.38
14.22	16.11	15.04	14.69	15.01 ± 0.40
13.87	16.07	11.80	13.27	13.75 ± 0.89

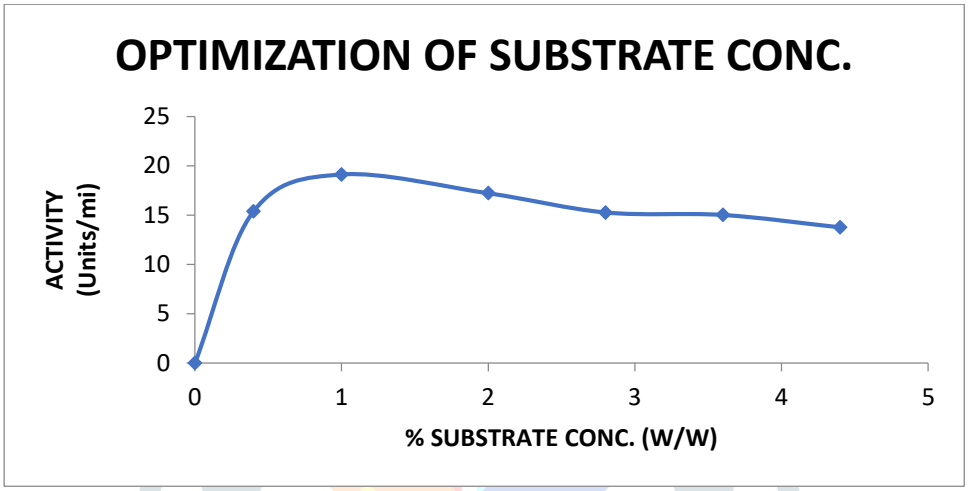

Figure 6: The activity of β-galactosidaseas a function of the lactose concentration (w/w)

Purification:

In the ATPS, it is possible to extract the enzyme at a rather high purity in one step. The reason for this is a combination of the very high partition coefficient (concentration ratio top/bottom) for β-galactosidase in the PEG-potassium phosphate system used and the tendency of other proteins to partition to the bottom phase. The mechanism of protein behavior in a PEG-salt two-phase system has been suggested by Busby and Ingham. It suggests that the two-phase system provides an opportunity for proteins to lower their chemical potential by escaping into the bottom salt phase when the PEG concentration in the top phase is increased (Busby et al., 1980). In a PEG solution of the necessary concentration, they would be precipitated. The reasons why β-galactosidase remains in a PEG solution are unclear. One possible explanation could be hydrophobic interaction since β-galactosidase has been reported to exhibit hydrophobicity when studied with hydrophobic chromatography (Morrow et al., I975).

When a particular enzyme prefers one of the phases very strongly, the way β-galactosidase prefers PEG, the enzyme can be concentrated in one phase by keeping the volume small and the major part of the remaining proteins can be recovered in the opposite, relatively large phase, although their partition coefficients are not very far from one. Thus a considerable purification ratio can be obtained in one single extraction step. This fast and efficient isolation step in an aqueous two-phase system, using inexpensive chemicals and conventional techniques, provides a suitable basis for further purification where technique like chromatography, gel filtration, and ultra filtration could be used.

Table 9: Shows total protein in PEG phase where mostly β-galactosidase present

ATPS	Total protein in crude, µg	Activity in crude, Units/mg cells	Total protein in PEG phase, µg	Activity of PEG phase, Units/ml	Volume of PEG phase, ml	Location of cells
1	3438.22	443.76	2414	374.82	2.05	I
2	3438.22	443.76	1266.67	556.85	1.5	I
3	2048.51	127.00	709.23	157.77	0.5	B
4	3438.22	443.76	1389.15	576.23	1.35	B
5	3438.22	443.76	901.74	371.59	2.6	B

Table 10: Purification of β-galactosidase from *E.coli* K-12 (To each composition, 1 g of disintegrated cell suspension was added, giving the final concentration of 11.75 % (wet wt.). The total weight of the system in each experiment was 3.257 g.)

ATPS	Potassium phosphate [% (w/w)]	PEG 4000 [% (w/w)]	Specific activity in enzyme solution, Units/mg	Specific activity in PEG phase, Units/mg	Specific activity in Salt phase, Units/mg	Fold Purity
1	12.98	6.14	128.9	318.31	27.74	2.5
2	14.51	6.75	128.9	659.42	12.29	5.11
3	15.57	7.33	62.00	222.52	30.53	3.1
4	16.05	7.98	128.9	559.99	9.43	4
5	9.90	10.75	128.9	107.14	16.64	----

The fold purity was increased when PFG concentration is increased from 6.14 to 6.75%but afterward it decreased compare to it at 6.75%. The maximum fold purity was found to be 5.11 where cell debris was collected at the inter-phase of the system. At higherconcentration of PEG and at lowestconcentration of salt, there was no purification of the enzyme. In this case the cell debris was collected at the salt phase.

CONCLUSION

1. Optimum production parameters giving maximum production of β-galactosidase for the inoculum (18 hr age, 2 ml suspension/100 ml media i.e. 2% v/v, Absorbance 0.56 at 610 nm) are,
   - Optimum Time: 18 hr
   - Optimum Temperature: 37 ^{0}C
   - Optimum pH: 7.2
   - Optimum Substrate Concentration: 1 %

   The maximum activity for 2% inoculum was found to be 27 Units/ml at these production parameters.

2. The maximum fold purity was found at system two where PEG-4000 & Phosphate salts were at 6.75% &14.51% composition respectively.

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