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Comparative Study of Soluble, Immobilised and co-immobilized Glucose Isomerase Enzyme in the production of High Fructose syrup from molasses

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ABSTRACT
This paper represents production of high fructose syrup. For the production of High Fructose Syrup a cheap cane molasses was selected. The isomerisation of glucose to fructose experiments was conducted using soluble and immobilized glucose Isomerase enzyme. The experimental investigations covered a wide range of parameters value such as isomerisation temperature, pH range and incubation period and enzyme concentration. The paper has comparative study of soluble and immobilized Glucose Isomerase enzyme.
Keywords:- High fructose syrup, immobilization, Molasses, Sweetener

I. INTRODUCTION

The high fructose syrup is one of the most revolutionary products in food science as the main consumers of HFS are beverages, baking, and confectionary, dairy and canning industries. It is preferred by food industry because it does not pose the problem of crystallization as sucrose does**.** The conversion of glucose to fructose by using free glucose Isomerase enzyme is not economical because the high cost free enzyme when used in the solution is not recoverable.

This problem could be overcome if the enzyme is immobilized. The aim of immobilization is to obtained superior enzyme for application which is highly active, stable and has appropriate specificity. The advantage of immobilized glucose Isomerase enzyme, it is used for longer duration due to its longer life in the support material and the ability to be reused. The optimum temperature of the immobilized enzyme in most of the cases increases thus allowing the isomerisation to be carried out at elevated temperatures which in turn favour higher fructose production. The temperature stability of the immobilized enzyme is higher. The pH stability of immobilized enzyme is more.

II. MATERIAL AND METHODS

2.1 Material

The chemicals, which were used for various studies, were of AR/LR grade and procured from M/S S.D. fine chemicals and M/S Loba Chemie Pvt. Ltd. Glucose isomerise and bio Invertase were obtained from M/S Biocon India limited, Bangalore (India) .The ion exchange resin, Indion 850 used for immobilization was kindly donated by ion – Exchange India ltd.

2.2. Preparation of reagent

2.2.1. Resorcinal Reagent- 1 gm resorcinol and 0.25 g thiourea were dissolved in 100 ml glacial acetic acid

2.2.2. Dilute HCl-Five parts of conc. HCl was mixed with one part of distilled water

2.2.3. Standard fructose solution-**50** mg of fructose or molasses was dissolve in 50 ml distilled water and diluted 10 times for a working standard.

2.3. Procedure-

a. From the stock solution of fructose or molasses 0.2, 0.4, 0.6, 0.8, 1 ml was taken in five test tubes and the volume made up to 2 ml with distilled water.

b. 1ml resorcinol and 7 ml diluted HCl were added in each tube.

c. A blank was set along with the working standard and then all the tubes were heated in a water bath at 80° c for 10 minutes. After 10 minutes the test tubes were cooled.

The color was read at 520 nm by spectronic 21 spectrophotometer within 30 minutes. The amount of fructose liberated in different reactions was determined

2.4. IMMOBILAZION OF ENZYMES USED IN STUDY

Immobilization of Glucose Isomerase Enzyme

The two ml (0.297 U/ml) of Glucose Isomerase enzyme was added to 500 mg of resin Indion 850 equilibrated with 0.01 M Sodium phosphate buffer, pH 7.5.The suspension was placed at 4°c for 2hr with occasional stirring to facilitate the adsorption of the enzyme on the resin. After 2hr, the supernatant liquid was removed, and the resin was washed twice with 2.0 ml of the same buffer and used.

2.5. Co-immobilization of Invertase and Glucose Isomerase Enzyme

Two ml (0.297 U/ml) of Glucose Isomerase enzyme and two ml of Invertase enzyme (1.2U/ml) was added to 500 mg of resin Indion 850 equilibrated with 0.01 M Sodium phosphate buffer, pH 7.5.The suspension was placed at 4°c for 2hr with occasional stirring to facilitate the adsorption of the enzyme on the resin. After 2hr, the supernatant liquid was removed, and the resin was washed twice with 2.0 ml of the same buffer and used.

III. COMPARATIVE STUDIES OF GLUCOSE ISOMERASE WITH FREE AND IMMOBILIZED ENZYME

3.1. Effect of Temperature and pH on Fructose Production

For optimization of temperature 1ml glucose (2.5M), 1 ml$MgCl_2$ (0.05M) were taken in 100 ml conical flasks and 50 mg immobilized beads were added and incubated in a shaker at 20, 30, 40, 50, 60, 70, 80 and 90°c at 220 rpm for 30 minutes and for soluble enzyme, 1 ml glucose (2.5M), 1ml $MgCl_2$ (0.05 M) and 1 ml enzyme were taken in conical flasks and reactions are carried out in similar manner. At the end of the incubation period reaction was stopped with $HClO_4$ and fructose was estimated as described earlier.

For optimization of Ph, 1 ml glucose (2.5M) and 1 ml $MgCl_2$ (0.05M) were taken in 100 ml conical flask.50 mg of immobilized beads were added (beads were immobilized in different buffers from 4 to 9) and incubated in shaker at 220 rpm for 30 min. the reaction was stopped with 0.5 ml $HClO_4$ and fructose was estimated in the reaction mixture. For soluble enzyme 1 ml glucose, 1 ml $MgCl_2$ and 1ml glucose isomerism enzyme were taken and fructose production was determined in the same manner as with the immobilized enzyme.

3.2. Fructose Production at Different Time Intervals Using Immobilized and Soluble Enzyme

10 ml of glucose containing 500 mg immobilized beads and 1ml $MgCl_2$ were taken in four different flasks and rotated at 220 rpm at 45°c. Sample from the incubation mixture were withdrawn at 30 minutes interval for the analysis of fructose produced. The treatment was carried out for 120 minutes. For soluble isomerism enzyme were taken in four different conical flasks and reaction conditions were similar as in the case of immobilized enzyme.

IV. REUSE OF IMMOBILIZED ENZYME

1 ml of glucose containing 50 mg immobilized glucose isomerise beads (0.297U/ml) and 1 ml $MgCl_2$ was rotated at 220 rpm at 45°c for 30 minutes. After 30 minutes the reaction was stopped with 0.5 ml $HClO_4$ and fructose was estimated. After completion of the process, the beads were withdrawn from the incubation mixture and washed with distilled water. These beads were again reused with fresh incubation mixture.

4.1. Effect of incubation period on Fructose production from molasses by co-immobilized glucose Isomerase and Invertase before and after clarification of molasses

10 ml molasses (ten times diluted) containing 500 mg co-immobilized beads and 10 ml $MgCl_2$ were taken in four separate conical flasks and rotated at 220 rpm at 45°c. Samples from the incubation mixture taken out at 30 minutes intervals, reducing sugar and fructose produced were estimated. The treatment was carried out for 120 minutes.

4.2. Effect of Temperature on fructose production from clarified Molasses using co-immobilized enzyme

10 ml clarified molasses (ten times diluted) ,10 ml $MgCl_2$ and 500 mg co-immobilized beads were taken in conical flasks and incubated for 30 minutes at40,45,50,60,70,80 and 90°c.After 30 minutes the reaction was stopped and fructose produced was estimate

V. RESULT AND DISCUSSION

5.1. Optimization of operational parameters of the soluble enzyme

Several experiments were performed to optimize the conditions for the soluble enzyme. The effect of temperature, pH, and concentration of enzyme and substrate were evaluated to establish the optimum conditions of the soluble enzyme. The effect of temperature on soluble enzyme was studied at different temperatures from 25° to 80°c. Results are shown in figure. It can be seen from fig that enzyme activity was maximum at 45°c with 3.5 units. Different glucose concentration was tested for conversion in to fructose. The kinetic constants of soluble enzyme were evaluated from Line Weaver Burk plot. Figure shows that Km for soluble enzyme was 3.87. The effects of concentration of enzyme on enzyme activity were studied and results are shown in figure. Different concentrations of enzyme (3.2 U/ml) from 0.2 to 1.0 ml were taken at 45°c for 30 minutes and pH 7.0 it shows that maximum activity of 2.6 units with 1 ml enzyme.

5.2. COMPARATIVE STUDY OF GLUCOSE ISOMERIZATION WITH FREE AND IMMOBILIZED ENZYME

Effect of Temperature on Fructose Production by Soluble and Immobilized Enzyme

For observing the effect of temp on fructose production by soluble and immobilized enzymes

eight different temperatures of 20, 30, 40, 50, 60, 70, 80, 90°c were tested. The optimum temperature for the soluble enzyme was found to be 60°c with 4.2 enzyme units in comparison to the immobilized enzyme showing an optimum activity at 70°c with 8.2 units.

TABLE 1 – Comparative study of Fructose production at different temperature by soluble and. immobilized enzyme.

Sr. No.	Incubation period (◦c)	Enzyme units		% Fructose Conversion	
		Soluble	Immobilized	Soluble	Immobilized
1	20	1.2	2.2	1.5	2.6
2	30	2.6	3.7	3.1	4.4
3	40	3.7	4.8	4.5	5.7
4	50	4.2	5.8	5	7
5	60	4.5	7.2	5.4	8.6
6	70	3.5	8.2	4.2	9.5
7	80	2.8	6.5	3.4	7.8
8	90	2.4	5.9	2.9	7.1

Production of Fructose at different pH by Soluble and Immobilized Enzyme

The effect of pH immobilized and soluble glucose isomerise were studied at different pH from 4 to 9. Maximum enzyme units were obtained at pH 8 for immobilized glucose isomerise and pH 7 for soluble enzyme

TABLE 2: Comparative study of Fructose production at different pH by soluble and immobilized Enzyme.

S.No.	PH	Enzyme units		% Fructose Conversion	
		Soluble	Immobilized	Soluble	Immobilized
1	4	1.2	2.2	1.5	2.6
2	5	2.4	3.7	2.9	4.4
3	6	3.5	5.5	4.2	6.4
4	7	4.5	6.5	5.4	7.8
5	8	3	7.5	3.6	9.1
6	9	2.1	5.9	3.6	7

Effect of incubation period on fructose production by immobilized and soluble Glucose Isomerase

The time of incubation is a significant parameter in determining enzyme activity and its conversion percentage. To study the effect of time of incubation reaction was carried out using glucose isomerise enzyme in soluble and immobilized states up to 120 minutes. Immobilized enzyme showed maximum conversion of 5.5% at 120 minutes

Reuse of immobilized beads

The stability of glucose isomerise beads was tested by examining the percent conversion of fructose in 30 minutes repeatedly for 10 cycles. It is evident that at the end if the first run the conversion was 8%. This value remained constant till the 3rd run followed by a fall up to 6.6% and 5.7%.

TABLE 3: Effect of incubation period on immobilized and soluble glucose isomerase

Sr. No.	Incubation period (min.)	Enzyme units		% Fructose Conversion	
		Soluble	Immobilized	Soluble	Immobilized
1	30	3.1	5.5	3.7	6.6
2	60	3.8	6.9	4.6	8.3
3	90	4.2	8.2	5.3	9.8
4	120	4.6	9.2	5.5	11.1

Effect of incubation period on Fructose production from molasses by co-immobilized glucose Isomerase and Invertase before and after clarification molasses

Fructose production from molasses by co-immobilized glucose Isomerase and Invertase was tested. For this both clarified and unclarified molasses was used. Fructose and total reducing sugar were estimated in clarified molasses after treatment at different time intervals while only fructose was estimated in unclarified molasses. The incubation was carried out up to 120 minutes. Clarified molasses showed maximum reducing sugar of 35.2 g/100ml and fructose 23.2 g/100 ml at 120 minutes. In the present work the glucose to fructose ratio 30, 60, 90 and 120 minutes of 50 mg of immobilized beads containing 0.297 units of glucose Isomerase and 1.2 units of Invertase was taken. Incubation time were 60:40, 59:51, and 35:65 respectively

TABLE 4: **Effect of incubation period on Fructose production from molasses**

Sr .No.	Incubation period	Reducing sugar liberated in gm/100ml molasses	Fructose liberated in gm/100 ml molasses before clarification	Fructose liberated in gm/100 ml molasses after clarification
1	20	25.2	8	10.4
2	60	30.2	11.2	12.6
3	90	33.2	14.2	17.2
4	120	35.2	20	23.2

Production of fructose from Molasses using co immobilized beads at different temperature

For observing the effect of temperature on fructose production by co-immobilized enzyme seven different temperature of 40, 45, 60, 70, 80 and 90°c were tested for 30 minutes. The optimum temperature for the co-immobilized Invertase and glucose isomerise was found to be 80°c with maximum fructose production of 23.2g/100ml as shown in table.

TABLE 5: Production of fructose from molasses using co immobilized beads at different temperatures. (Enzyme concentration: Glucose isomerise 0.297 units and Invertase 1.2 units.)

Sr .No.	Temp (°c)	Enzyme units	Fructose liberated in g/100 ml molasses in 30 min
1	40	17.7	9.6
2	45	22.2	12
3	50	28.2	15.6
4	60	32.9	17.8
5	70	37	20
6	80	42.5	23.3
7	90	30	16.4

REUSE OF CO-IMMOBILIZED INVERTASE AND GLUCOSE ISOMERASE ENZYME

The co-immobilized Invertase and glucose isomerise was used repeatedly for the production of fructose for 8 cycles. For this purpose the enzyme concentration was doubled. Thus 0.594 units of glucose isomerise and2.4 units of Invertase enzyme were used. It can be seen from fig that co-immobilized enzyme beads could be used for a number of cycle. The data indicates that production of fructose were almost same in first three cycles (37.8%). Till 6th cycle the performance of the resin was appreciable with 35.2%, 34.8% and 32.4% production of fructose. The activity of enzyme was checked at 30 minutes interval for 8 cycles.

TABLE 6: Repeated Use of co-immobilized Invertase and Glucose Isomerase Beads at 45°c

Sr .No.	No. of cycles	Fructose liberated in g/100 ml molasses
1	1st	37.8
2	2nd	37.8
3	3rd	37.8
4	4th	35.2
5	5th	34.2
6	6th	32.4
7	7th	29.1
8	8th	24.6

Activity of the Co-immobilized Enzyme Beads on Preservation at 4°c at Different Time Intervals

To examine the stability of co-immobilized enzyme on keeping, the activity of the enzyme was checked at 10 days intervals for 50 days. The results are shown in fig 15. It can be seen from fig that the production on the 1st, 10th, 20th, 30th, and 40th, days were 37.8%, 37.8%, 35.0% and 34.8%. This suggests that the beads could be used till 40 days without much loss in activity.

VI. CONCLUSION

This paper indicates that the soluble enzyme showed maximum activity at temperature 45°c and pH 7.0. The conversion of glucose to fructose was determined and Value for soluble enzyme was found to be 3.87. Cheap ion exchange resin such as Indion 850 was chosen for immobilization. The immobilization with Indion was very simple and economical process. The immobilized beads showed maximum activity at 60°c, pH 8.0, after 120 minutes incubation. The temperature and pH optima of the immobilized enzyme were thus raised compared to the soluble enzyme. The used resin could be easily regenerated by washing with distilled water and could be reused for several cycles. The activity was reduced after 10 cycles. This investigation thus suggests that immobilized glucose isomerase can be used economically for fructose production. As immobilization raises the temperature optimum of the enzyme system, conversion at higher temperature may lead to better results. For the production of high fructose syrup a cane molasses was selected. It was clarified with lime and superphosphate before use. Maximum fructose conversion was found to be 23% at 80°c temperature in 30 minutes using co –immobilized Invertase and glucose Isomerase (GI 0.297 units and Invertase 1.2 units). Co immobilization increases the temperature optimum for the beads further. By doubling the

enzyme concentrations in the immobilized beads the conversion could be significantly enhanced. The maximum of 37.8% conversion was achievable in 30 minutes and appreciable amount of fructose was produced when the beads were recycled. The beads were fairly stable on storage for over a month. This investigation thus suggests that co-immobilized glucose Isomerase and Invertase can be used economically for fructose production using molasses. AS co-immobilization raises the temperature optimum of the enzyme system, conversion at higher temperature may lead to better results.

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