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BIOSYNTHESIS OF GLUCOSE ISOMERASE BY LOCALLY ISOLATED *STREPTOMYCES ALBUS* IN SHAKE FLASKS

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Streptomyces albus WRL–7 was locally isolated for the synthesis of intracellular enzyme glucose isomerase in shake flasks. Of all carbohydrates: xylose induced maximum enzyme synthesis. The optimum level of xylose was1.0 % w/v and the amount of enzyme produced was 3.36 U/ml. The xylan, polymer of xylose, containing agricultural by-products such as corn cobs, wheat bran, rice bran or cotton seed hulls were evaluated as such or their hydrolysate for enzyme synthesis. The productin of glucose isomerase was induced by all of them but enzyme production was better in the presence of corn cobs or wheat bran. Effect of partial replacement of xylose by glucose was also investigated but the results of enzyme formation were not encouraging. Effect of the addition of nitrogen sources such as peptone, yeast extract, beef extract, malt extract CSL or casein on the production of glucose isomerase was also studied. Peptone or CSL was found to be 1.0 % i.e., 3.21 U/ml. The production of enzyme in the presence of inorganic nitrogen sources, however, was greatly effected. The mineral nutrition of the selected culture was also inestigated. The presence of Mg^{++} and Co^{++} was found to be essential for enzyme synthesis.

INTRODUCTION

The enzyme glucose isomerase is being commercially exploited for the production of high fructose corn syrup as sucrose substitute for food and pharmaceutical industries [1,2]. Marshall and Kooi [3] first reported the ability of *Pseudomonas hydrophila,* grown on xylose medium to convert D-glucose to D-fructose. Since then a large number of microorganisms, capable of producing glucose isomerase, have been isolated by several workers. Among them *Streptomyces* species have been extensively studied as a source of enzyme including the following [4–9], *S. albus, S. phaeochoromogenes, S. bikinies, S. flavorence, S. fradiae, S. venezuelae.* Other organisms include [10–15]. *Aerobacter cloacae, A. aerogens, Bacillus coagulance, Lactobacillus brevis. Erchericia intermedia* and *Actinoplanes missouriensis.*

Most of the production methods were not economical, because glucose isomerase formaion required xylose an expensive enzyme inducer. However, Takasaki [4] isolated an organism which could produce good quality of glucose isomerase when grown on a medium containing xylan or xylan containing materials such as corn cobs, wheat bran or cotton seed hulls. Dworschack, *et al,* [16] patented methods for the production of glucose isomerase from *Streptomyces* ATCC 21175 using acid hydrolysate of corn cobs or cotton seed hulls as a source of xylose. Xylan containing agricultural products such as wheat bran corn hulls, corn cobs, and cotton seed hulls are abundantly available in the country and no such study has been conducted earlier on their utilization for the production of glucose isomerase.

The present study describes the production of glucose isomerase by locally isolated culture of *Streptomyces* species, on xylose-mineral salt medium in shake flasks. Further work has also been carried out using agricultural wastes as source of xylose, such as, wheat bran, corn cobs. Cotton seed hulls or rice bran, that stimulates the enzymes formation in shake flasks.

MATERIALS AND METHODS

The *Streptomyces* species were isolated from soil by dilution plate technique as describes by William and Davis

[17]. The isolation medium contained (g/l) starch, 10.0; casein 10.0; NaCl, 2.0, K_2HPO_4, 2.0. $MgSO_4 \cdot 7H_2O$, 0.05, $CaCO_3$, 0.02; $FeSO_4.7H_2O$ 0.01 and agar 20; pH 7. The medium also contained (g/l) sodium peniciliin G 0.001 and nystatin, 0.05 to suppress the growth of bacteria and mould respectively. Soil samples were dispersed in sterile water, plated on the agar media and incubated at 28–30° for ten days. Sharp and distinct colonies were isolated and transferred to agar slants.

Screening Test: Cultures of *Streptomyces* species were grown on a screening medium containing (g/l) D-xylose, 10.0; peptone 10.0 yeast extract 5.0; $K_2HPO_4.7H_2O$ 1.0; pH 7.0; The medium was distributed in 10 ml amount in 50 ml cotton wool plugged conical flask and sterilized for 15 min at 15 lb. After inoculation with test organism from the slant, the shake flask cultures were incubated for 48 hr at 30° on rotary shaker. The cells were collected by centrifugation and tested for enzyme activity described below.

Culture Maintenance: The selected cultures were maintained on agar medium consisting of (g/l) D-xylose (in the form of H_2SO_4 hydrolyzate of corn cobs) 5; Dextrose, 5; peptone 10; yeast extract 5.0; K_2HPO_4 3.0. $MgSO_4.7H_2O$ 1 agar 25; pH 7.0 in deionized water. The culture were incubated at 30° for five days and then kept at 4° for later use. Subsculturing was made after every three or four weeks.

Vegetative Inoculum. The inoculum medium consisting of (g/l) D-xylose (as hydrolyzate of corn cobs) 10.0; peptone, 10.0; yeat extract, 5.0; K_2HPO_4, 3.0, $MgSO_4.7H_2O$ 1.0, dissolved in deionized water (pH 7.0). To avoid cramelization, sugar solution was sterilized separately and combined aseptically before inoculation. 20 ml of the above medium was taken in 100 ml conical flask, sterilized and inoculated with a loop full of spores from slant culture and incubated at 30° on a rotary shaker for 48 hr. This seed culture was used as inoculum for shake flask experiment. The inoculum size was 5 % v/v.

Fermentation. The fermentation was carried out in duplicate in 250 ml conical flaks, containing 50 ml of fermentation medium (g/l):– D–xylose (in the form of acid hydrolyzate of corn cobs) 10; cron steep liquur 2.0; $MgSO_4.7H_2O$ 1.0 $CoCl_2.6H_2O$, 0.2 and K_2HPO_4 2.0. The pH of the medium was adjusted to 7.0. the culture medium were autoclaved at 121° for 15 min and inoculated with seedculture and incubated at 30° on a rotary shaker at 180 rpm for 48 hr.

Extraction of Enzyme. The cells from 100 ml fermented broth were harvested by centrifugation at 5°. Washed twice with cold deionized water and finally suspended in 20 ml 0.05M phosphate buffer (pH 7.0) 0.1% cetyl trimethyl ammonium bromide was added and the content were incubated with mild agitation at 40° for 6 hr. The cell debris after cooling in ice was removed by centrifugation. The supernatant was used as enzyme extract.

Determination of Enzyme Activity. Glucose isomerase acitivity was determined according to the modified method of Dische and Boren freund [18]. The enzymic reaction mixture contained 0.1 ml of IM glucose, 0.1ml of 0.1M $MgSO_4.7H_2O$, 0.1 ml of 0.01 M, $CoCl_2.6H_2O$, and 0.5 ml of 0.2 M phosphate buffer pH 7 and one ml of cell suspension or cell free extract. The final volume of the enzyme assay mixture was made up to 2 ml with distilled water. The mixture incubated at 70° for one hour, the reaction was stopped by adding 2 ml of 0.5 M perchloric-acid. The coagulaed protein was removed by centrifugation. The supernatant after appropriate dilution was analyzed for fructose formed. One unit of glucose isomerase activity was defined as the amount of enzyme that produced 1 μ mol of fructose per min. under the above assay conditions.

Preparation of Hydrolyzate of Corn Cobs. 25 g of sun dried corn cobs was grounded to pass 100 mesh sieve, mixed with 250 ml of 0.2 N H_2SO_4 and autoclaved for 60 min at 15 lb. The mixture was filtered and the suppernatant was neutrilized with $BaCO_3$ and used as H_2SO_4 hydrolyzate of corn cobs. Xylose content of the hydrolyzate was determined according to modified method of Lane-Eyne [19].

RESULTS

Screening of Organisms. The data of Table 1 shows the synthesis of cell mass of various *Streptomyces* isolates and their capacity of glucose isomerase production in shake flasks. The results are the average of triplicate experiments. Of all the cultures investigated *Streptomyces* sp. WRL–7 produced maximum amount of enzyme glucose isomerase per unit mass. This culture was characterized and identified as *Streptomyces albus* WRL–7 according to Nomeura [20]. In Subsequent experiments the culture of *Streptomyces albus* WRL–7 was used throughout.

Size of Inoculum. Effect of adding difference size of inoculum (2–10 % v/v) on glucose isomerase formation in shake flask was investigated. The inoculum was in general 48 hr. old. The production of enzyme glucose isomerase was maximum with 4 % inoculum i.e. 3.56 U/ml, 42 hr after seed transfer (Fig.1).

Nutritional Studies. Effect of Carbon Sources: Effect of the addition of different carbon sources on the biosynthesis of enzyme was studied (Table 2). The initial concentration of sugar was kept at 1.0 w/v. Of all carbohydrates

Table 1. Production of glucose isomerase by locally isolated streptomyces species.

Culture No.	Enzyme activity U/ml broth	Dry cel Wt. mg/ml	Specific-activity U/mg/cell	Culture No.	Enzyme activity U/ml broth	Dry cell wt. mg/ml	Specific-activity U/mg/cell
WRL–1	0.34	4.18	0.08	WRL-23	0.86	4.12	0.20
WRL-2	0.37	4.44	0.08	WRL-24	nil	3.65	nil
WRL-3	nil	4.21	nil	WRL-25	0.27	4.23	0.06
WRL-4	nil	3.60	nil	WRL-26	nil	nil	nil
WRL-5	1.15	4.36	0.26	WRL-27	nil	4.23	nil
WRL-6	0.71	4.23	0.16	WRL-28	0.04	1.86	0.02
WRL-7	2.05	5.25	0.39	WRL-29	0.32	3.42	0.09
WRL-8	nil	5.15	nil	WRL-30	nil	2.84	nil
WRL-9	0.06	4.25	0.01	WRL-31	nil	nil	nil
WRL-10	nil	nil	nil	WRL-32	0.75	4.35	0.17
WRL-11	0.96	4.65	0.02	WRL-33	0.35	4.15	0.08
WRL-12	nil	5.16	nil	WRL-34	0.25	3.62	0.07
WRL-13	nil	nil	nil	WRL-35	1.25	5.20	0.24
WRL-14	0.27	5.05	0.05	WRL-36	0.96	5.15	0.19
WRL-15	1.08	4.86	0.18	WRL-37	0.82	4.86	0.17
WRL-16	0.02	4.32	0.01	WRL-38	0.15	3.84	0.04
WRL-17	nil	nil	nil	WRL-39	0.32	4.00	0.08
WRL-18	nil	2.32	nil	WRL-40	nil	2.65	nil
WRL-19	nil	1.86	nil	WRL-41	nil	nil	nil
WRL-20	0.26	4.35	0.05	WRL-42	0.02	1.18	0.01
WRL-21	0.06	3.36	0.01	WRL-43	0.10	2.19	0.04
WRL-22	nil	2.26	nil	–	–	–	–

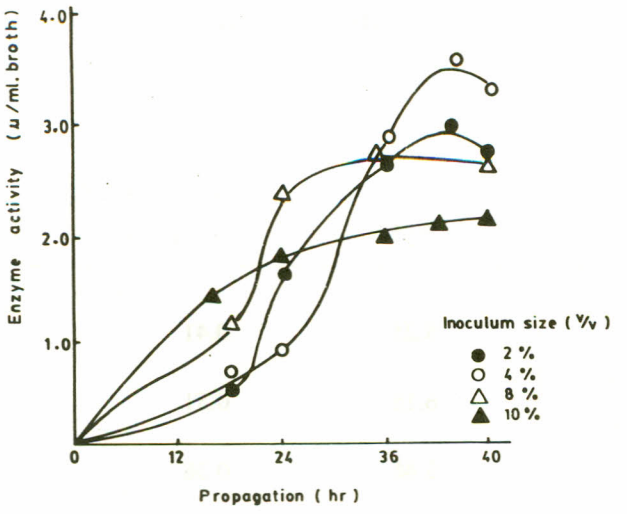

Fig. 1. Effect of inoculum sige on glucose isomerase formation by *S. albus* WRL-7.

added only xylose induced glucose isomerase i.e. 0.46 U/mg cell. The enzymic activity of the fermented broth, was 2.85 U/ml. The cell synthesis was greater with D-xylose and lowest in the presence of D-glucose.

The optimum level of D-xylose was also determined by adding different concentration of sugar i.e., 0.25 – 5.00 % w/v. The enzymic assay was carried out 48 hr after inoculation with seed culture. The activity of glucose isomerase was maximum in the presence of 2 % w/v xylose. Further increase in sugar levels however, decreased enzyme production by shake culture.

Effect of Xylan Containing Substances on Enzyme Synthesis. The xylan containing agricultural by-products such as corn cobs, corn husks, wheat bran and cotton seed hulls were evaluated as enzyme inducer by adding them to the fermentation medium (Table 2). The amount of these materials added to the basal medium was 1–4 % w/v. The enzymic activity of fermented broth was determined 48 and 56 hrs after inoculation. The enzyme formation was maximum in the presence of 3–4 corn cobs, wheat bran or cotton seed hulls i.e. 2.25 U/ml. Further effect of adding acid hydrolysate of corn cobs or cotton seed hulls on glucose isomerases formation was investigated (Table 3). The concentration of xylose was 0.5 – 2.0 % w/v. The enzymic activity in the broth or specific activity of cells was better in the presence of 1.0 % w/v xylose. Further increase in the amount of xylose did not show significant effect on enzyme production.

Effect of partial replacement of xylose by glucose on enzyme formation was also studied (Table 4). The concentration of total sugar was kept at 1.0 % w/v. The production of glucose isomerase in the fermented broth or specific activity of cells was decreased with the increase

Table 2. Effect of xylan containing material on the growth and glucose isomerase formation from *S. albus* WRL–7.

Kind of xylan source	Concentration %	Encyme yield U/ml broth 48 hr	56 hr
Corn cobs.	1	1.25	1.46
	2	1.80	1.85
	3	2.30	2.51
	4	2.15	1.90
Wheat bran.	1	0.86	1.25
	2	1.51	1.46
	3	2.00	2.15
	4	2.15	2.26
Cotton seed hull	1	1.00	1.36
	2	1.85	2.15
	3	1.76	2.35
	4	0.82	1.10
Rice bran.	1	0.36	.65
	2	0.52	1.15
	3	0.95	1.72
	4	1.85	2.10

in the concentration of glucose. The inhibitory effect was significant at 2.0 % glucose and further increase in glucose concentration reduced both the enzymic activity of fermented broth or specific activity of the cells.

Effect of Nitrogen Sources on Enzyme Formation. (a) Organic: The addition of nitrogen sources to the fermentation medium play a significant role on enzyme synthesis by microbes. Evaluation of various nitrogen sources on the production of glucose isomerase by *Streptomyces sp.*

Table 3. Effect of concentration of acid hydrolyzate on growth and glucose isomerase formation by *S. albus* WRL–7.

Acid hydrolyzate	Concentration of xylose supplied %	Enzyme yield μ/ml broth	Dry cell wt mg/ml broth	Specific activity μ/mg cell
Corn cobs.	0.5	2.16	5.25	0.41
	1.0	3.12	6.15	0.51
	1.5	2.00	5.46	0.36
	2.0	0.56	3.92	0.14
Cotton seed hull	0.5	2.00	5.15	0.39
	1.0	2.92	5.56	0.52
	1.5	2.16	5.44	0.40
	2.0	0.65	4.33	0.15

Table 4. Effect of partial replacement of xylose by glucose on the production of glucoseisomerase and growth by *S.albus* WRL–7.

Xylose concentration %	Glucose concentration %	Enzyme activity μ/ml	Dry cell wt/mg/ml	Specific activity Specific activity μ/mg cell
1.0	0.0	3.25	5.36	0.60
0.9	0.1	3.10	5.35	0.58
0.8	0.2	1.26	4.88	0.26
0.7	0.3	1.22	4.57	0.26
0.5	0.5	1.15	4.32	0.26

was carried out (Table 5). Of all nitrogen sources, the enzymic activity was better in the presence of corn steep liqour (CSL), yeast extract or peptone. The levels of nitrogen sources, added to the medium were 1–2 % w/v. The enzymic activity was maximum in the presence of CSL. In subsequent experiments, therefore, CSL was used as source of nitrogen in production medium. The optimum level of CSL was also determined by adding different concentration of CSL to the fermentation medium. The production of glucose isomerase however, was observed maximum in the presence of 1% w/v CSL i.e., 3.21 U/ml.

(b) Inorganic :Effect of inorganic nitrogen sources such

Table 5. Effect of organic nitrogen sources on the production of glucose isomerase and growth by *S. albus* WRL–7.

Nitrogen sources	Concentration %	Enzyme activity μ/ml	Dry cell weight mg/ml	Specific activity μ/mg
Peptone	1	2.45	5.56	0.44
	2	3.55	6.35	0.56
Yeast extract	1	1.82	4.76	0.38
	2	2.46	5.48	0.49
Beef extract	1	1.22	4.26	0.28
	2	1.64	4.82	0.34
Malt extract	1	1.74	4.34	0.40
	2	1.62	4.56	0.34
Corn steep liquor	1	3.20	6.26	0.50
	2	3.32	5.96	0.53
Casein	1	0.97	3.34	0.29
	2	1.35	4.38	0.31

as $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$, NH_4Cl, KNO_3, $(NH_4)_2HPO_4$ and urea on glucose isomerase formation was also studied in shake flasks. The nitrogen sources were added to the level of 0.5 % w/v in the fermentation medium. Of all nitrogen sources, however, diammonium hydrogen phosphate gave better result of enzyme synthesis i.e., 1.85 U/ml. The enzyme synthesis, however, was better with organic nitrogen sources as described earlier.

Effect of Metal Ions. Effect of the addition of metal ions as Mg^{++}, Co^{++}, Mn^{++}, Fe^{++}, Zn^{++}, Cu^{++} on glucose isomerase formation by *Streptomyces albus* was studied in shake flasks. The metal ions were added at the levels of 1.0 mM and 5.00mM to the basal medium. The biosynthesis of glucose isomerase was increased by adding $MgSO_4.7H_2O$ (5mM) and $CoCl_2.6H_2O$ (1.0mM) in the fermentation medium. At higher concentration of $CoCl_2.6H_2O$ (5.0 mM) the production of enzyme was greatly affected. In the presence of Mn^{++}, Fe^{++}, Zn^{++}, Cu^{++}, Ni^{++}, however the enzyme formation was negligible.

The optimum levels of both Mg^{++} and Co^{++} were further determined by adding varying amount of these metal ions to the basal medium. The concentrations of Mg^{++} and Co^{++} were varied from 1.0 – 8.0 mM and 0.25 – 2.5 mM respectively. The optimum level of Mg^{++} was found to be 10 mM and enzymic activity was 2.31 U/ml, further increased in the concentration of Mg (6.0mM) did not increase enzyme formation but at 8mM the glucose isomerase production was reuduced to 50 %.

The optimum concentration of Co^{++} was 1 mM and the enzyme produced was 3.65 U/ml. Glucose isomerase synthesis, however was greatly affected at 2.5 mM of Co^{++} i.e., 0.85 U/ml. Effect of the addition of Co^{++} (1mM) at different times of inoculation on enzyme formation was also investigated. Glucose isomerase synthesis was maximum when Co^{++} was added 18 hrs after the inoculation of vegetative cells i.e., 3.65 U/ml. The dry cell weight was 6.75 mg/ml.

Rate of Fermentation. Fig.2 shows the time course of glucose isomerase fermentation by *Streptomuces albus* in shake flasks. The parameters studied were changes in pH, cell mass, xylose utilization and enzyme formation.

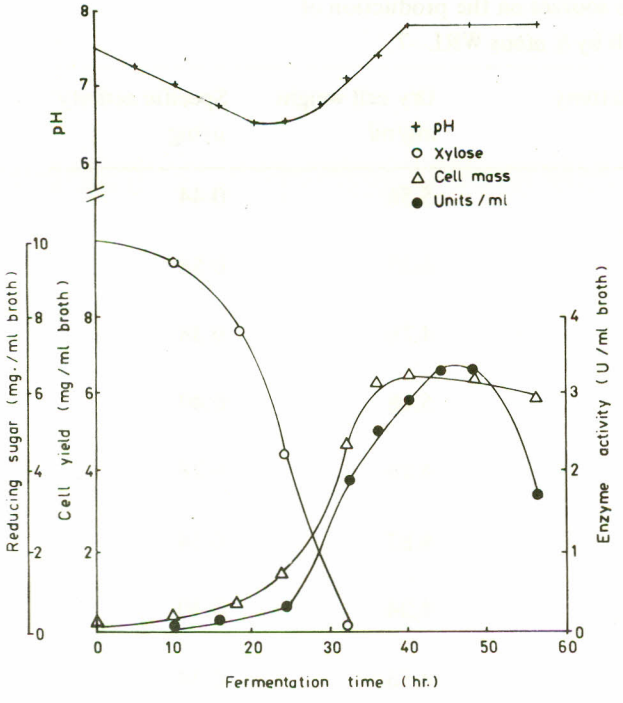

Fig. 2. Time course of glucose isomerase fermentation.

Highest level of glucose isomerizing activity was reached in 44 – 48 hr after inoculation (3.2 U/ml). Xylose was completely utilized during 30 hr of fermentation. The maximum cell yield of 6.3 mg/ml occured 36 – 40 hr after inculation. The pH of the broth at first decreased from 7.0 to 6.5 and then increased to 7.9 when the growth was in active phase and thereby remained constant till the end of fermentation.

DISCUSSION

The present work describes the synthesis of inducible enzyme glucose isomerase by locally isolated culture of *Streptomyces albus* WRL–7. The nutritional requirements of the culture were determined in shake flasks. Effect of the addition of various sugars in the basal medium was investigated on cell synthesis and enzyme formation. The cells grew in the presence of all sugars but glucose isomerase was induced only in the presence of xylose. The optimum concentration of xylose was 1.0 per cent w/v and the amount of enzyme produced was 0.46 U/ml cells. The amount of cells produced was the lowest in the presence of glucose. The production of enzyme was also carried out in the presence of xylan, polymer of xylose, containing agricultural by-products or their acid hydrolysate.

Both rates and amount of enzyme formation were better in the presence of acid hydrolysates of all agricultural resources, acid hydrolysates of corn cobs produced maximum enzyme synthesis i.e., 3.12 U/ml. Partial replacement of xylose by glucose resulted in the decrease of enzymic activity.

Effect of the addition of various nitrogen sources, both organic and inorganic, on the production of glucose isomerase was also examined. The enzymic activity was maximum in the presence of peptone and CSL i.e., 3.55 and 3.32 U/ml respectively. CSL being cheaper nitrogen source was preferred for scale up studies. Inorganic nitrogen sources, however, gave poor yield of glucose isomerase in the fermented medium. The reason for low enzymic yield was may be due to the lowering of pH of the medium. The addition of urea, however, resulted in the increase of pH i.e., above neutral and this also reduced the production of enzyme. The optimum temperature and pH for enzyme synthesis were 30^{o} and 7.5 respectively. The growth of cells and enzymic activity were greatly affected below pH 6.0 or above 8.5. The presence of Co^{++} and Mg^{++} were essential for the synthesis of glucose isomerase. Their optimum levels were found to be 0.001M and 0.01M respectively. Glucose isomerase formation and cell growth were greatly affected by adding Fe^{++}, Zn^{++}, Cu^{++}, Ni^{++} or Mn^{++} to the basal medium. The addition of Co^{++} to the basal medium 18 hr after inoculation with vegetation cells gave better result of enzyme production and its addition after wards however, showed no stimulatory affect on enzyme production. The production of glucose isomerase reached maximum 44 – 48 hr after inoculation.

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