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EFFECT OF CARBON AND NITROGEN SOURCEAND MEDIA OPTIMIZATION FOR THE PRODUCTION OF GLUCOSE ISOMERASE FROM *ARTHROBACTER SPECIES* (5327)

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ABSTRACT

The present study is to isolate and screening of glucose isomerase from *arthrobacter species (5327)* for industrial application of fructose. *Arthrobacter* has emerged as an in exhaustive treasure for a wide range of enzymes like glucose isomerase. Glucose isomerase screening was performed by three different methods such as plate assay using xylose containing peptone yeast agar medium, protein estimation by barfods test. *Arthrobacter species (5327)* were grown on peptone yeast extract broth, the cell biomass was harvested then disrupted by ultrasonic disintegrator and centrifuged; cell free extracts were used for glucose isomerase activity. Fixing of carbon and nitrogen source in a minimum concentration was assayed and the shake flask experiments are done which has proved numerous *Arthrobacter species (5327)* has been produced glucose isomerase activity, particularly it has been proved superior performing for glucose isomerase production and can be used for industrial application of fructose production.

KEYWORDS: *Arthrobacter Species*, Glucose isomerase, Enzyme assay.

INTRODUCTION

In the early 1960s, two routes to make fructose from glucose are explored. The first one, a chemical route of converting glucose to fructose under alkaline conditions is studied extensively but none of the processes developed is commercialized mainly because of the difficulty involved in the prevention of degradation products, which affected the product quality, reduced the sweetness and also resulted in flavour and colour rendering the product unsuitable for its intended application, the food industry. The second route, enzymatic isomerisation of glucose to fructose is the only alternative left out and with the identification of a number of microorganisms as being capable of producing the necessary enzyme, xylose isomerase is being practiced commercially now a days. Some of the commercially viable organisms are Streptomyces, Actinoplanes, Bacillus and *Arthrobacter Species (5327)* [Higginus *et al.*, 1976].

The chemical conversion of glucose to fructose has been known for the past 100 years and constitutes one of a group of reactions collectively known as the Lobry de Bruyn-Alberda van Ekenstein transformation. These reactions are usually carried out at high pH and temperature [Blow, *et al.*, 1992]. The possibility of producing the reaction is nonspecific and leads to the formation of non metabolizable sugars such as psicose and other undesirable colored products. It is difficult to attain a fructose concentration of more than 40% by this method. Moreover, chemically produced fructose has off flavors and reduced sweetness, which cannot be easily remedied. Therefore, it cannot be used commercially.

Glucose isomerase/ Xylose isomerase (EC. No. 5.3.1.5) is a commercially important enzyme that plays an essential role in microbial sugar metabolisms. The greatest market for glucose isomerase is in food industry; particularly catalyzes two important reactions such as reversible isomerization of D-glucose to D-fructose and D-xylose to Dxylulose for possible application to ethanol production from hemicelluloses [Seyhan Tukel and Alagoz, 2008]. The production of rare monosaccharides, such as L-glucose, L-fructose, L-ribose, L- lyxose L-galactose by glucose isomerase, recently has received much interest to potential health and medical benefits [Jokela *et al*., 2002]. Glucose isomerase catalyzes the conversion of glucose to fructose, which has significant value in the production of High Fructose Corn Syrup (HFCS). The world market for glucose isomerase is US$1billion and about 100,000 tons of glucose isomerase is made annually in global scale [Vandamme *et al*., 2005]. Thus, there is a great demand for glucose isomerase globally. Increasing demand for refined sugar, coupled with high cost of production and awareness of the adverse effects of sucrose and invert sugar consumption on human health, fructose for acceptable sucrose substitutes. Fructose has the benefit an equal

sweetener level, 10-20% cheaper than sucrose and less caloric, D-fructose plays an essential role in a diabetic sweetener because fructose is slowly reabsorbed by the stomach and does not pressure the glucose level in blood [Cowan, 1996; Barker, 1976]. HFCS is used as sweetener in soft drinks and other food products where fructose replaces beet and cane sugar. Technical advantage of HFCS is the good solubility of glucose and fructose compared to sucrose and the lesser tendency to crystallize in a wide range of food products[BhosaleandRao1996].

MATERIALS AND METHOD

REVIVAL OF THE ORGANISM

The organism has been obtained from National Chemical Laboratory (NCL)-Pune. From the Mother culture the Organism *Arthrobacter Species (5327)* has been sub cultured in Nutrient Agar.

ESTIMATION OF BIOMASS

The subculture a loopful of culture was inoculated into 50ml sterile Nutrient Broth and incubated for 24 hours at 37c. After incubation 5 ml was transferred into 45 ml of sterile Nutrient Broth and incubated for 16 hours at 37c. From the 16 hour culture 2 ml was taken and diluted with distilled water.(0.2 ml broth +0.8 water, 0.4 ml broth +0.6, water, 0.6 ml broth +0.4 water, 0.8 ml broth +0.2water). Optical Density was read at 600nm. The remaining 48 ml was centrifuged at 10,000 rpm for 15 minute. The supernatant was discarded and the pellets were washed with Phosphate buffer then Dried in hot air oven and the dry weight was estimated.

pH OPTIMIZATION

From the subculture a loopful of culture was inoculated into 50ml sterile Nutrient Broth and incubated for 24 hours at 37c. After incubation 5 ml was transferred into 45 ml of sterile Nutrient Broth and incubated for 16 hours at 37c. From the 16 hour culture 5 ml was transformed to each flask of 45 ml sterile Nutrient Broth with different pH (pH 4, pH 6, pH 7, pH 7.5, pH 10, pH 12). The inoculated Flasks were incubated at 37 c for 24 hours in the shaker at 70 rpm. After incubation Optical Density was read at 600nm.

ENZYME ASSAY

The production medium used for studies of glucose isomerase production included (Peptone 10-g/l, Yeast extract-5g/l, K_2HPO_4-3g/l, $MgSO_4.7H_2O$- 1g/l, Glucose - 3 g/l and Sucrose-2 g/l (sterilized separately) as a source of carbon nutrition. The initial pH was adjusted to 6.8 with 1 M Hcl. The experiments were carried out in 250-ml Erlenmeyer flasks, each containing 50 ml of the medium, incubated with shaking (180 rev/min) at 25 °C for 24h. The cultured cells were separated by centrifugation, washed twice with distilled water and used for determination of cell dry weight, intracellular glucose isomerase activity and preparation of cell-free enzyme extracts.

PREPARATION OF CELL FREE ENZYME EXTRACT

To prepare cell-free extracts of glucose isomerase a thick paste of washed cell was placed in a mortar, frozen with liquid nitrogen, disrupted mechanically and extracted with K Phosphate buffer (pH 7.8) on a shaker for 1 h. In order to remove cell debris the suspension was centrifuged at 7000 rpm for 15 min and the supernatant was used as the enzyme solution.

EVALUTION OF CELL DRY WEIGHT

Dry cell mass was estimated by weighing washed cells after drying at 105 °C for 24 hrs.

DETERMINATION OF FRUCTOSE

To 0.1 ml of a solution containing fructose, 0.1 ml of 12% cysteine hydrochloride and 6 ml of 75% sulfuric acid(a mixture of water and concentrated sulfuric acid in the ratio of 5:1)are added and immediately afterwards 0.2% of a 0.12 per cent alcoholic solution of carbazole. The mixture is shaken and left standing at room temperature. The colour turns from blue to Purple. The color developed was read at 670 nm against reagent blank.

PROTEIN ESTIMATION

The total amount of protein present in the culture filtrate was measured by Braford's method.

FERMENTATION PROCESS

The medium was coated and the inoculum load was added aseptically into the vessel. The aeration was maintained at 5 vvm and the agitation speed was maintained at 200 rpm. At Every interval of 2 hours 10 ml of sample was withdrawn. The pH and contamination was checked. The sample was centrifuged at 10,000 rpm for 10 minute and the supernatant was used for Enzyme assay and the supernatant was used as a Blank for the determination of Biomass. Optical Density was read at 600nm.

FIXING INNOCULAM LOAD

From the subculture a loopful of culture was inoculated into 50ml of Production Media and incubated for 24 hours at 37◦C in a shaker at 70 rpm. After incubation 10 ml was transferred into 90ml of Production Media and incubated for 16 hours at 37c in a shaker at 70 rpm. The Production Media of 40, 41, 4,2 43, 44, 45, 46, 47 ,48, 49 ml was made up to 50 ml with 16 hour culture and incubated for 24 hours at 37c in a shaker at 70 rpm. Optical Density was read at 600nm.

SELECTION OF CARBON AND NITOGEN SOURCE

The Carbon source selected was Sucrose and the Nitrogen source selected was Peptone and yeast extract for the production of Glucose Isomerase from *Arthrobacter Species (5327)* (Edwarden ,*et al.,* 2005)

FIXING THE MAXIMUM AND MINIMUM CONCENTRATION OF CARBON AND NITOGEN SOURCE

In the Production Media along with Glucose, Sucrose was added as Carbon source in the concentration of 0.5g/l, 1 g/l, 2 g/l, 4g/l, 8g/l, and 16g/l. The fixed inoculam load was added to all the concentration of Sucrose. The incubation hour was fixed from the Maximum growth time of Fermentor. After Incubation at 22 hours in shaker at 70 rpm the Optical Density was read at 600nm to determine the Maximum and Minimum concentration of Sucrose. In the Production Media, Nitrogen source Corn steep liquor was first replaced with Peptone in the Concentration of 0.5g/l, 1 g/l, 2 g/l, 4g/l, 8g/l, and 16g/l. the fixed Inoculam load was added to all the concentration of Maltose. The incubation hour was fixed from the Maximum growth time of Fermentor. After Incubation at 20 hours in shaker at 70 rpm the Optical Density was read at 600nm to determine the Maximum and Minimum concentration of Peptone.

Then the Beef extract was replaced with Yeast extract in the Concentration Of 0.5g/l, 1 g/l, 2 g/l, 4g/l, 8g/l, and 16g/l. the fixed inoculam load was added to all the concentration of Maltose. The incubation hour was fixed from the Maximum growth time of Fermentor. After Incubation at 20 hours in shaker at 70 rpm the Optical Density was read at 600nm to determine the Maximum and Minimum concentration of Yeast extract. The Maximum concentration was considered as (-1) and the Minimum concentration was considered as (+1).

PLAKETT BURMAN DESIGN AND MEDIA FORMULATION

The Plackett-Burman design comprise of two level screening designs. These designs allow for the study of factors with n+ 1 trial. In this design, low and high factor settings were coded as -1 and +1 respectively. Each factor was tested an equal number of times at its low and high settings. The table was design for a combination of low and high settings for 3 variables in 8 experiments. The following were the variables considered Sucrose, peptone and Yeast extract.

TABLE 2: OPTICAL DENSITY Vs pH

S.No	pH	TRIPLET VALUE			Average
		1	2	3	
1	4	0.157	0.158	0.157	**0.15**
2	6	0.230	0.230	0.230	**0.23**
3	7	0.682	0.682	0.682	**0.68**
4	7.5	0.774	0.772	0.774	**0.77**
5	8	0.714	0.714	0.714	**0.71**
6	10	0.124	0.124	0.124	**0.12**
7	12	0.091	0.091	0.091	**0.19**

Screening was done when the investigator was faced with a large number of factors and was unsure which settings are likely to produce optimal or nearly optimal responses. A number of tools may be used to help, access the significance of each process factor. These include P-values, normal plots and Pareto charts. The most common means of accessing significance is the P-value. The P-value is the probability that the magnitude of the parameter estimate is due to random process variability. A low P-value indicates a real or significant effect and provides a baseline for screening some factors as critical and less important. Out of these, only the most effective components with positive effects can be selected for further study while showing large negative effects may be dropped in all future experiments.

TABLE 1: PLACKETT BURMAN DESIGN FOR 3 VARIABLES

EXPT.NO	X1	X2	X3
1	-1	-1	**-1**
2	+1	+1	**-1**
3	-1	+1	**-1**
4	-1	+1	**+1**
5	+1	-1	**+1**
6	+1	-1	**-1**
7	-1	-1	**+1**
8	**+1**	**+1**	**+1**

SHAKE – FLASK EXPERIMENTS

5ml of 24 hours inoculum of the organism was added to 41 ml of production media in 250 ml Erlenmeyer flasks. The range and levels of the test variables were followed according to the experimental design. The pH value of the medium was adjusted before sterilization by adding 1 N Hcl or 1 N NaoH. After incubation of 20 hours the enzyme activity and Biomass was estimated. All the experiments were carried out in Triplicate and the average values were reported.

RESULTS

The organism was grown at different pH and the optimum pH for the growth of the organism was found to be 7.5 and was shown in Table: 2. the difference between the biomass and pH was shown in fig: 1

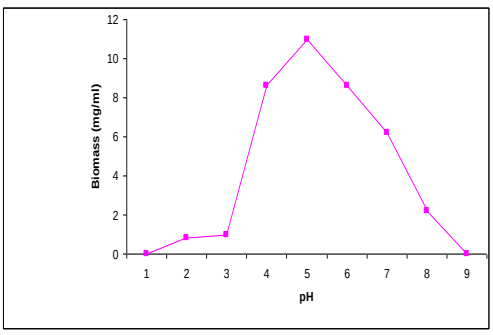

Fig: 1: BIOMASS VS pH

The life cycle of the organism has been studied and it was found that the organism enters the lag phase at 2nd

hour and the log phase extends up to 22nd hour and it is described in figure: 2.

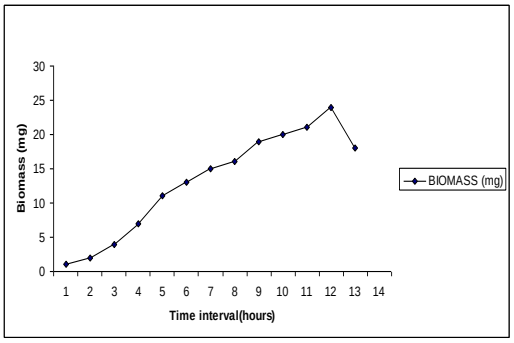

Fig: 2: LIFE CYCLE OF THE ORGANISM

The production of glucose isomerase was also found to be maximum at 12th hour which was shown in figure: 3

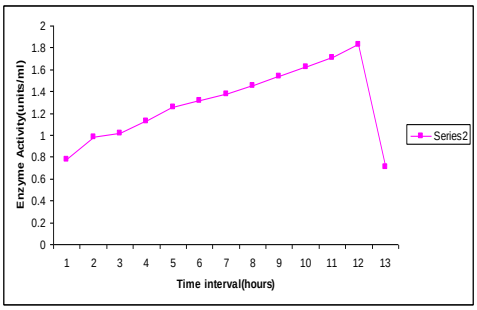

Fig: 3 GLUCOSE ISOMERASE ACTIVITY AT VARIOUS TIME INTERVALS

The total protein was determined by Braford's method and estimated as 150mg/ml. The enzyme activity at 22nd hour was 1.83 (IU/ml).

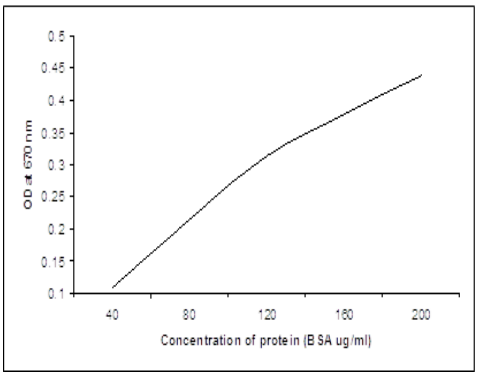

Fig: 4: PROTEIN ESTIMATION

Sucrose was used as carbon source and the minimum and maximum concentration in which the enzyme produced was found to be 1.0-4.0g. Peptone was used as nitrogen source and the minimum and maximum concentration in which the enzyme produced was found to be 0.5-8.0g. Yeast extract was used as nitrogen source and the minimum and maximum concentration in which the enzyme produced was found to be 4.0-8.0g.The level of the nitrogen sources and carbon sources are shown in Table:3.

TABLE 3: CARBON SOURCES AND NITROGEN SOURCES

	NITROGEN SOURCES				CARBON SOURCE	
S.NO	**PEPTONE (g/l)**	**OPTICAL DENSITYAt 600 nm**	**YEAST EXTRACT (g/l)**	**OPTICAL DENSITY At 600 nm**	**SUCROSE (g/l)**	**OPTICAL DENSITY At 600 nm**
1	0.5	0.214	0.5	1.239	0.5	**0.239**
2	1	0.218	1	0.198	1	**0.198**
3	2	1.115	2	1.115	2	**1.258**
4	4	1.284	4	1.284	4	**1.475**
5	8	1.306	8	1.203	8	**1.203**
6	**16**	**1.237**	**16**	**1.305**	**16**	**1.352**

TABLE 4: MINIMUM & MAXIMUM CONCENTRATION OF CARBON AND NITROGEN SOURCE

S.NO	SOURCES	MAXIMUM (g/l)	MINIMUM (g/l)
1	SUCROSE	4	**1**
2	PEPTONE	8	**0.5**
3	YEAST EXTRACT	4	**8**

Placket- Burmann designs and for the screening of 3 variables has been executed by conducting 8 trials. Based on the P-value and E-value of the response Peptone was found to be the best nitrogen source compared with Yeast extract.

E-Value was calculated as E-Value =Average of responses at high level - Average of responses at low level .P- Value was found out using student t-test.

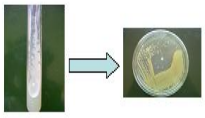

TABLE 5: ENZYME PRODUCTION FOR VARIOUS MEDIA COMPOSITION

S.NO	TRAIL	ENZYME PRODUCTION
1	T1	0.112
2	T2	0.070
3	T3	0.153
4	T4	0.073
5	T5	0.052
6	T6	0.459
7	T7	0.312
8	T8	**1.210**

The sucrose used also showed P & E value as 0.055 and 0.7234 respectively which indicates the used sucrose as the best one which was shown in Table 6.

TABLE 6: E-VALUES AND P-VALUES

VARIABLES	E-VALUE	P- VALUE	RANK
Sucrose	0.7234	0.055	**II**
Peptone	0.8217	0.027	**I**
Yeast extract	**0.0201**	**0.0721**	**III**

DISCUSSION

Our isolated strain was found to be the producer of GI. Next step was to design of medium for GI production; this was done by standard references of W.P. CHEN. The next work was on inoculums preparation, after that the production was started and completed in two days, the completion of production resulted into extraction of enzyme which was carried out by firstly separating biomass because our interest of enzyme was intracellular. This was done by spinning the fermentation medium at 12,000g and then homogenized to lyses the cells and enzyme was separated and purified by ammonium sulphate precipitation and then on dialysis (Misset *et al*., 1991). The extracted enzyme were checked for protein contents by barfods method and the concentration of unknown protein was found to be assayed for enzyme activity for the different parameters such as temperature and pH and the isolated enzyme found to be optimum at 80 and pH 7. Isomerase activity of enzyme was checked by the seliwanoff's test and the amount of fructose produced was found to be 700 mg/ml. From this work we had ultimately concluded that the our regions Streptomyces can also produced significant amount of glucose isomerase that can decrease cost of purchasing the other strains and also our strain can limit the temperature variation because of our climatic condition there is a no need to maintain the low and high temperature.

CONCLUSION

The optimum pH for the growth of organism was found to be 7.5 and the same was maintained throughout the study. The temperature was maintained at 37°C the organism at early log phase was used for the entire work. The organism has shown higher glucose isomerase activity at 22nd hour. Among the nitrogen source Peptone was found to be the best compared with Yeast extract. The carbon source was also found to be best therefore Sucrose and Peptone can be used for the further study of optimization.

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