CHAPTER -V

GLUCOSE ISOMERASE PRODUCTION BY

STREPTOMYCES SP. SB-AII4 AND STREPTOMYCES SP. SB-P1

5.1 INTRODUCTION

The production of Glucose Isomerase (GI) is done at industrial scale for HFCS production. GI is reported to be a thermostable enzyme produced by mesophilic as well as thermophilic organisms. The enzyme has been screened among a variety of bacterial species eg. Pseudomonas hydrophila, Lactobacillus brevis, Aerobacter aerogenes and Bacillus coagulans but most of the reports are on Streptomyces. These filamentous organisms produce majority of the enzymes in bulk amounts.

Most of the producers require xylose as an inducer for GI production but there are some exceptions. One, such organism, Actinoplanes missouriensis, can be cultivated in a relatively simple growth medium which is able to produce glucose isomerase with commercial potential and does not need xylose as an inducer. A. missouriensis produces an intracellular soluble glucose Isomerase [Gong et al., 1980]. Glucose isomerase production is also reported from non filamentous bacteria. Kwon et al., (1987) produced GI from alkalophillic bacilli; Ogbo and Odibo, (2007) reported the production of GI from Saccharococcus caldoxylosilyticus, Lee and Zeikus, (1991) from thermoanaerobic bacteria C. thermosulfurogenes and Thermoanaerobacter and Kovalenko et al., (2011) used a recombinant E. coli strain for GI production. Fan et al., (2011) produced GI from Thermoanaerobacter ethanolicus; Manhas and Bala, (2004) used Streptomyces sp. for GI production Sriprapundh et al., (2003) from Thermotoga neapolitana, Raykovska et al., (2001) from Streptomyces thermovulgaris. Hasal et al., (1992) has reported from Streptomyces sp. Sanchez and Smiley, (1975) from Streptomyces albus and Calik et al., (2009)
Glucose Isomerase Production by Streptomyces sp. SB-AII4 and Streptomyces sp. SB-P1

from *Bacillus thermoantarcticus*. **Givry and Duchiron, (2008)** produced GI using *Lactobacillus bifermentans*.

Most of the investigators have screened for GI production by submerged fermentation process [Manhas and Bala, 2004; Hasal et al., 1992; Lee and Zeikus, 1991]. The production media, production time and inoculation methods are different. The time required for production has a direct influence on the cost of the production process. A fast growing strain in optimally rich medium shall definitely give high yields. Biotechnical application of glucose isomerases depends on several criteria like easy fermentation of the bacterial strain, high cell yield, simple and high yield purification procedures, and long-term stability of the enzyme.

The extracellular release of the enzyme is also a point of consideration as it may be due to the autolysis of the mycelial cells. The extraction processes opted by investigators are usually treatment with a surface active agent or physical methods like ultrasonication [Gong et al., 1980]. The process of recovering enzyme from the fermented broth is also an important step. Low pH buffer offered higher enzyme recovery than did higher pH buffer.

Several trials have been done on *Streptomyces* sp for scaling up and industrialization of the process [Hasal et al., 1992; Thakur et al., 1986]. **Thakur et al., (1986)** observed that *Streptomyces fradiae*, a producer of glucose isomerase became denatured because of repeated subculturing. Therefore any strain to be taken up to industrial level must also be checked for it’s genetic stability.

At industrial level the glucose isomerase is immobilised as pure enzyme or whole cells for the production of HFCS [Kovalenko et al., 2011; Kim and Pyong-Su, 1992].

The technology for production of glucose isomerase can be designed by considering a few points in detail i.e. the growth requirements of the organism and the growth stage for the production of enzyme. The enzyme is used in the primary metabolism of the organism’s growth therefore it’s production may also
coincide with the exponential growth phase. The maximum accumulation of enzyme was observed by different investigators between 36 to 96 h.

The crude enzyme remains stable over a period of time when stored in a freezer, but the purified enzyme denatures upon freezing and thawing. The purified enzyme can be stored in a freezer with 40% glycerol. This enzyme solution does not freeze at the storage temperature of -20°C and is stable over several months without loss of activity. The glycerol does not affect the isomerization activity of the enzyme [Lee and Zeikus 1991].

It is known that the presence of substrate has a great influence on thermal stability of various enzymes. This is due to the fact that the binding of the enzyme with substrate and forming enzyme-substrate complex stabilizes the secondary and tertiary structure of the enzyme. Thermostability can also be explained by the amino acid composition of the enzyme. Hydrophobic amino acid residues predominate in the molecule of glucose isomerase which results in numerous hydrophobic interactions within the enzyme molecule. The non covalent bonds which maintaining the secondary and tertiary structure are responsible for stability of the enzyme to a greater extent than the disulphide bonds. The low content of methionine, tryptophan and absence of cysteine in glucose isomerase structure explicit the inability of these amino acids to form covalent bonds [Tashpulatova et al., 1991].

In order to use the organism’s metabolic machinery well, the organism’s choice of conditions have to be determined. The Streptomyces being slow growers take around six to seven days for growth; the time required for maximum accumulation of enzyme also has to be determined.

Glucose isomerase is an intracellular enzyme in majority of the cases but extracellularly producing organisms are also known [Manhas and Bala, 2004]. In the present study we are focusing on an extracellular GI producer. This is opted in order to reduce the production cost and time by eliminating the cell disintegration step. Majority of the sources for our isolates are compost pit samples which favors the probability of presence of a thermostable enzyme.
5.2 MATERIALS AND METHODS

5.2.1 Comparison for intracellular and extracellular enzyme production

*Streptomyces* are known to produce GI intracellularly as well as extracellularly. The selected isolates P1, V5, KB1, NPI2 and AII4 were screened for both the types of enzymes. The centrifuged supernatant was used as extracellular crude enzyme extract and intracellular enzyme was extracted by CTAB method [Chen and Anderson, 1979]. The fermented broth was centrifuged and biomass separated. The biomass was washed thrice with distilled water. The cell mass was suspended in 0.05 M sodium phosphate buffer (pH-7), containing 0.1% cetyl trimethylammonium bromide (CTAB). The enzyme was extracted for 2 h at 37°C in shaking condition. This was centrifuged at 5000 RPM for 10 mins and the supernatant was used as the intracellular enzyme extract.

5.2.2 Growth Curve for *Streptomyces* sp. SB - AII4

A set of ten 100 mL conical flasks containing 20 mL of Bennett’s broth (Appendix - I) were inoculated with *Streptomyces* sp. SB - AII4. All the flasks were incubated at 30°C and 120 RPM on orbital shaker. One flask was harvested after every 24 h. The biomass was separated from the nutrient medium by centrifugation and washed with distilled water. The biomass was transferred on a filter paper and kept for drying at 55°C. The dry weight was determined after 24 h.

5.2.3 Time Scale Analysis of *Streptomyces* sp. SB - AII4

Time scale analysis was performed in order to determine the fermentation period required for maximum production of glucose isomerase. *Streptomyces* sp. SB - AII4 was inoculated in seven flasks containing 20 mL of Bennett’s broth (Appendix - I). After a fixed interval of 24 h for 7 days a set of flasks were harvested and fermentation process was terminated. The increase in enzyme accumulation was determined by performing enzyme assay described in section 4.2.2.1. The centrifuged crude supernatant was used to measure
extracellular glucose isomerase. The intracellular glucose isomerase was extracted by CTAB method described in section 5.2.1.

5.2.4 Growth Curve for Streptomyces sp. SB - P1

Streptomyces sp. SB - P1 was inoculated in ten 100 mL flasks containing Bennett’s broth (Appendix - I) and incubated at 30°C and 120 RPM in orbital shaker. One flask was harvested everyday till the tenth day and dry mass determined as described in section 5.2.2.

5.2.5 Time Scale Analysis Streptomyces sp. SB - P1

Optimum incubation time required for maximum accumulation of extracellular glucose isomerase was determined from Streptomyces sp. SB - P1. The culture was inoculated in seven 100 mL conical flasks having 20 mL of Bennett’s broth (Appendix - I). The amount of extracellular glucose isomerase accumulated was determined after a regular interval of 24 h by performing the assay described in section 4.2.2.1.

5.2.6 Crude enzyme kinetics

Glucose isomerase produced by different organisms isomerises the substrate at different optimum conditions. The optimum conditions for GI activity extracellularly by Streptomyces sp. SB - P1 were determined.

5.2.6.1 Effect of Substrate Concentration on GI activity

The effect of concentration of substrate on enzyme activity was determined to estimate the optimum concentration of substrate for maximum enzyme activity. The $K_m$ and $V_{max}$ values were determined. A series of test and control tubes were prepared containing range of substrate (glucose) concentration between 0.25 Moles to 2 Moles. The reaction mixture and rest of the components were added in the same ratio in all the tubes as already described in section 4.2.2.1.
5.2.6.2 Effect of Enzyme Aliquot on GI activity

The optimum volume of enzyme extract to be used was determined. In the series of tubes crude enzyme aliquots ranging from 0.05 mL to 0.4 mL were added. This is done in order to quantify the minimum volume of enzyme required for efficient activity. The assay was carried out keeping all other components in the proportion described in section 4.2.2.1.

5.2.6.3 Effect of Temperature on GI activity

The influence of temperature on the isomerising efficiency of extracellular glucose isomerase produced by *Streptomyces sp. SB - P1* was studied. The reaction mixture was prepared in a series of tubes as described in section 4.2.2.1 and incubated at different temperatures in water bath, 50, 55, 60, 65, 70, 75, 80 and 85°C.

5.2.6.4 Effect of pH on GI activity

The effect of pH on glucose isomerase activity was determined. The assay was performed according to the details given in section 4.2.2.1. The reaction mixture was prepared in buffers of different pH ranging from 4 to 11. Acetate buffer was used for pH 4 and 5 (Appendix - II). Phosphate buffer was used for pH 6 and 7, Tris for pH 8 and 9 and Glycine buffer for pH 10 and 11 (Appendix - II).

5.2.6.5 Effect of Reaction period on GI activity

The effect of reaction period on the activity of enzyme was determined. The reaction mixture for assay was prepared according to the details given in section 4.2.2.1 and the tubes were incubated for different time periods from 15 minutes to 120 minutes. The reaction was terminated in one set of tubes after every 15 minutes and the fructose accumulated was estimated by Cysteine Carbazole method [Dische and Borenfreund, 1951].
5.3 RESULTS AND DISCUSSION

The experiment was run in triplicates to reduce the chances of error and the means were used to observe final results for all the experiments. The primary screening for glucose isomerase, mentioned in Chapter – IV yielded five good producers [P1, V5, KB1, NPI2 and AII4] of the enzyme. The organisms are reported to produce glucose isomerase extracellularly as well as intracellularly. The selected cultures were checked for production of both the categories of enzyme.

5.3.1 Comparison of Intracellular and Extracellular production of Glucose Isomerase

The primary screening of the isolates revealed P1, V5, KB1, NPI2 and AII4 to be the highest producers of the enzyme. Streptomyces sp. SB - P1 was found to be the maximum extracellular GI producer and Streptomyces sp. SB - AII4 the maximum intracellular producer. The extracellular and intracellular GI summed together shall give high final yields but the procedure and time involved in extraction of the intracellular enzyme definitely increases the cost of the process. The idea behind this study is to develop a technology which requires minimum input and gives maximum results. The amount of intracellular enzyme produced by P1, V5, KB1 and NPI2 was quiet less as compared to AII4. The extracellular and intracellular amount of enzyme produced by Streptomyces sp. SB - AII4 summed together was not significantly higher than the extracellular enzyme produced by Streptomyces sp. SB - P1. The highest enzyme yield we observed was 2.8 U/mL of the fermented broth as compared to a wide range of yield reported by earlier researchers [Bhosale et al., 1996] from 1 U/mL to 35 U/mL. This high yield is definitely obtained after standardization of production media, process parameters and lots of strain improvement steps. The titer of 2.8 U/mL is a fair enough start for optimisation of production technology and standardization of downstream processing protocol. The extracellular and intracellular enzyme yields by five isolates are graphically represented in Fig. 5.1.
Glucose Isomerase Production by Streptomyces sp. SB-AII4 and Streptomyces sp. SB-P1

Fig. 5.1: Comparison of Extracellular and Intracellular GI production by five best isolates.

5.3.2 Growth Curve for Streptomyces sp. SB - AII4

The appearance of biomass in the medium started after 24 h and increased successively. The growth of Streptomyces sp. SB - AII4 appeared in the form of small beads in clear transparent medium which is a characteristic feature of Streptomycetes [Williams et al., 1989]. The number and size of beads increased as the incubation time increased. The biomass showed a progressive increase till 5th day of incubation but later on the growth was not much. The organism seems to enter the stationary stage after 5th day of incubation. The growth of the isolate is reported in the form of dry weight and graphically presented in Fig. 5.2.
5.3.3 Time Scale Analysis for *Streptomyces sp. SB - AII4*

The extracellular enzyme production for AII4 could be detected only after 48 h of incubation. The accumulation of extracellular as well as intracellular enzyme increased till 96 h and later on it started decreasing. The results for extracellular GI production by *Streptomyces sp. SB – AII4* are presented in Fig. 5.3.

The enzyme started accumulating intracellularly after 24 h. The flasks harvested on successive days showed increase in the enzyme accumulation till 96 h. The organism entered in to the late stationary and thereafter the decline phase must have decreased the enzyme production. As prevalent from the growth curve also there is no substantial increase in the biomass which means that with the decrease in surviving cells the enzyme produced is also decreasing. The highest production observed by *Streptomyces sp. SB – AII4* was in 96 h as also reported by Dhungel *et al.*, (2007) from psychrotolerant *Streptomyces sp.* but Paik and Dewey, (1980) reported GI production by *Streptomyces sp.* in 72 h and Chen *et al.* (1979) observed optimum production time of 36 h by *Streptomyces flavogresius*. There are reports of good enzyme accumulation by Streptomycetes between 24 to 48 h also by Manhas and Bala,
Glucose Isomerase Production by Streptomyces sp. SB-AII4 and Streptomyces sp. SB-P1

(2004) and Hasal et al., (1992). The results for intracellular GI production by Streptomyces sp. SB – AII4 are presented in Fig. 5.3.

![Graph showing production of extracellular and intracellular GI at different intervals by AII4.](image)

**Fig. 5.3: Production of Extracellular and Intracellular GI at different intervals by AII4.**

**5.3.4 Growth curve for Streptomyces sp. SB - P1**

The growth for Streptomyces sp. SB – P1 was determined by measuring the dry weight of the biomass. The biomass increased till the sixth day of incubation progressively but there was no significant increase in the dry weight beyond this. The growth of organism must have ceased due to depletion of nutrients and accumulation of waste metabolites. The results are shown in Fig. 5.4.
5.3.5 Time scale analysis for *Streptomyces sp. SB - P1*

Production was terminated in a set of flasks after every 24 h and the broth was harvested to determine the amount of enzyme produced. The accumulation of enzyme increased till 96 h of incubation. Further incubation did not support the increase in productivity of glucose isomerase. This enzyme is produced by the organisms for utilization of available sources such as glucose or xylose as nutrients therefore the production will definitely start as the growth starts. As the number of bacterial cells goes on increasing the number of enzyme molecules shall increase, therefore high biomass accumulation will result in good enzyme yield [Bok et al., 1984]. The production of glucose isomerase is reported by Hasal et al., (1992) in 24 h, Manhas and Bala, (2004) in 48 h whereas Chen et al., (1979) observed the production in 72 h and Chou et al., (1976) in 96h by Streptomycetes. Maximum accumulation of enzyme in our case was observed in 96 h of incubation as also reported by Srinivasan et al., (1983) for extracellular production by *Chainia* species. Further incubation resulted in loss of enzyme activity. Gong et al., (1980) reported maximum production in 120 hours. The decrease of the enzyme activity during the stationary phase may be explained by the detrimental effects of acidic pH or some by-products formed in the
medium. Presence of proteases may also breakdown the enzyme present in the medium. The GI activity during seven days period is graphically depicted in Fig. 5.5.

![Graph showing GI activity over time](image)

**Fig. 5.5: Production of Extracellular GI at different intervals by Streptomyces sp. SB - P1**

### 5.3.6 Crude Enzyme Kinetics

The isolate producing highest amount of glucose isomerase extracellularly was picked up for further studies. We selected the extracellular producer in order to decrease the cost and time duration required for production by eliminating the cell disintegration step. The isolate *Streptomyces sp. SB - P1* was selected to develop further production technology as it is an extracellular glucose isomerase producer. Optimisation of conditions for enzyme activity is required in order to achieve maximum productivity of the process. The crude enzyme extract is assayed for parametric optimization for enzyme production and media designing, therefore crude extract was used for optimizing enzyme assay parameters. The optimum activity parameters have been worked out by various researchers and majority of the glucose isomerases have been found to be efficient at neutral or alkaline pH and in a range of 60°C to 80°C. Very few reports are available for the acidophilic optima of the enzyme.

#### 5.3.6.1 Effect of Substrate Concentration on GI activity

The effective substrate concentration to get maximum rate of enzyme reaction was determined. A series of tubes with increasing substrate
concentration in the range of 0.25 Moles to 2 Moles were prepared. The enzyme activity increased successively till 1.25 Molar concentration of the substrate after which there was no substantial increase. Lineweaver Burk plot [Fig. 5.7] was prepared and the $K_m$ value was calculated to be 1.24 M for glucose. The $K_m$ values reported for glucose by different researchers range from 0.22 M to 1.33 M [Raykovska et al., 2001; Bhosale et al., 1996; Kasumi et al., 1981; Gong et al., 1980] and $V_{max}$ observed is 3.68 U/mL. The results are graphically represented in Fig. 5.6.

![Fig.5.6: Effect of substrate (glucose) concentration on GI activity](image)

![Fig. 5.7: Lineweaver Burk plot](image)
5.3.6.2 Effect of enzyme aliquot on activity

The influence of increasing enzyme aliquot on GI activity was determined. This was done to find out the optimum amount of enzyme required for isomerising a given amount of substrate. The crude extract obtained after centrifugation of the fermented broth was used for this analysis. The activity increased till the tube containing 0.25 mL of aliquot but further increase was negligible. To avoid the unnecessary wastage of enzyme 0.2 mL can be considered as the optimum amount required for the analysis. The results are shown in Fig.5.8.

![Fig. 5.8: Effect of enzyme aliquot on GI activity](image)

5.3.6.3 Determination of optimum temperature for GI activity

Temperature activity profile was carried out by studying the isomerisation ability of the enzyme in a range from 50°C to 85°C. The effect of temperature on isomerisation is presented in Fig. 5.9. The enzyme is substantially active between 60°C to 75°C. The maximum isomerisation was achieved at 70°C. Our culture *Streptomyces sp. SB - P1* has been isolated from compost pit where extensive metabolic activities are going on as lots of plant and animal remains are present. This indicates the temperature optima for the isolate on higher side. The activity was quiet less at temperature lower than 60°C. Enzymes from different sources may have same temperature optima as...
stated earlier for optimum pH. Although many bacterial species have been checked for GI but the maximum share is occupied by *Streptomyces*. The isolates of *Streptomyces* from hot springs exhibited maximum activity at 70°C [Manhas and Bala, 2004; Al-Tai et al., 1987], *Streptomyces* from some other sources isomerised to their maximum at 80°C and 85°C [Dhungel et al., 2007; Chou et al., 1976; Bandlish et al., 2002]. GI also has been reported by *Bacillus* having 85°C as temperature optima [Lama et al., 2001]. Thermophilic strain of *Streptomyces thermovulgaris* 127, var. 7-86 performed best at 70°C [Raykovska et al., 2001]. Majority of the glucose isomerases have temperature optima on the higher side.

![Fig.5.9: Determination of optimum temperature for GI activity by *Streptomyces* sp. SB-P1](image)

### 5.3.6.4 Determination of optimum pH for GI activity

The effect of pH from 4 to 11 was studied for maximum enzyme activity. The enzyme was found to be substantially active between pH 6 to 9, whereas the maximum activity was observed at pH 7. The highest isomerisation was achieved at neutral pH which is an industrially beneficial result. This will
enable us to run the process conveniently and no extreme pH conditions are required. Bandlish et al., (2002) reported highest activity for Streptomyces marinus at pH 7 whereas Chou et al., (1976) reported their Streptomyces isolates exhibited a broad range activity from pH 7 to 8. A thermophilic strain Streptomyces thermovulgaris 127, var. 7-86 isolated by Raykovska et al., (2001) exhibited maximum activity at pH 8. Manhas and Bala, (2004) reported their Streptomyces isolates picked up from soil and water samples near hot springs to have acidic optimum pH and Dhungel et al., (2007) reported the Streptomycetes isolated from soil samples of Mt. Everest to have pH optima of 6.9. Kasumi et al., (1981) reported Streptomyces griseofuscus having optimum activity at 8.5. This kind of variation in the sources of the organisms having similar pH optima indicates the conserved structure of the enzyme with extended temperature stability. The results of effect of pH on GI activity are presented in Fig. 5.10.

![Fig. 5.10: Determination of optimum pH for GI activity by Streptomyces sp. SB - P1](image)

**5.3.6.5 Determination of suitable Reaction period for GI activity**

The time required for isomerisation is an important criterion for this enzymatic process. The optimum temperature at which the enzyme catalyses the reaction most effectively is considerably high. Although the enzyme is thermostable and high temperature favors the equilibrium towards fructose but
development of coloured impurities by incubation at high temperature for long periods can be avoided. The suitable reaction period was found to be 60 mins beyond which there was a decrease in the fructose estimated in the tubes. This is also affected by the thermostability of the enzyme. The results are graphically represented in Fig. 4.11.

![Graph showing GI activity vs time](image)

**Fig. 5.11: Determination of suitable Reaction period for GI activity**

### 5.4 CONCLUSIONS

The glucose isomerase is reported to be intracellular in majority of cases. The extraction process requires complicated methods for the extraction of the enzyme. Therefore we want to concentrate on an organism which is an extracellular enzyme producer. As we have already stated that we require an indigenous economically viable technology for commercial scale production, the use of extracellular enzyme shall decrease the production cost and time by avoiding the cell disruption step.

Some GI producers release the enzyme in the production medium as a result of autolysis. This is the appropriate time when we can recover the maximum enzyme without using any disruption method.
The biomass increase was observed continuously for 10 days but there was a progressive increase till 4th day which is associated with enzyme accumulation, this also indicates that the organism moves to it’s stationary phase where the increase in enzyme accumulation ceases. The decrease in the enzyme level in later days must be due to the proteolytic activities going on in the medium and decrease in the number of surviving cells in the decline phase of organism’s life cycle which can compensate the loss.

The comparison done between the intracellular GI producer *Streptomyces sp. SB – AII4* and extracellular GI producer *Streptomyces sp. SB – P1* has made this clear that the intracellular and extracellular yield of *Streptomyces sp. SB – AII4* summed together is not significantly higher than the extracellular GI produced by *Streptomyces sp. SB – P1* alone. Therefore developing a production technology by using *Streptomyces sp. SB – P1* shall be cost effective and the further studies will be concentrating on extracellular production.

The $K_m$ value for glucose was observed to be 1.24 M and $V_{max}$ was 3.86 U/mL which might differ when the enzyme is purified. The optimum range for high enzyme activity was broad between 60°C to 75°C which is a beneficial result from the point of view of industrialization where narrow range maintenance of physiological conditions like temperature and pH is critical and expensive. The optimum temperature determined for enzyme activity was 70°C at 7 pH.