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Comparative Study of Extraction Methods for Intracellularly Produced Glucose Isomerase by *Streptomyces sp. SB – AII$_4$*

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Abstract

An array of diverse kinds of Actinomycetes was screened for production of Glucose isomerase. Isolates were analyzed for the production of extracellular and intracellular glucose isomerase. The isolate Streptomyces sp. SB – AII$_4$ was identified as the highest intracellular GI yielding strain. The isolate produced GI extracellularly as well as intracellularly. The optimum fermentation period required for GI production was 96 hours at 30˚C and 100 RPM. A comparative analysis was done for maximum extraction of intracellular enzyme. Treatment with CTAB and Ultrasonication were found to be efficient methods for extraction of glucose isomerase.

Keywords: Streptomyces, glucose isomerase, intracellular, extraction, CTAB.

Introduction

Glucose isomerase (GI) has an ever-increasing demand because of its use as a low calorie sweetener and the production of high fructose corn syrup (HFCS)[1]. The reversible reaction of glucose into fructose, which is catalyzed by glucose isomerase, has great market value. GI finds major application in HFCS and ethanol production. HFCS is used in medicated syrups, beverages, baking, canning, and confectionary items as a sweetening agent. It is produced by processing corn starch to glucose and then glucose is isomerized to produce high percentage of fructose[2,3]. In the first step, cornstarch is treated with amylase to convert polysaccharide into monosaccharide. In the next step, Glucose Isomerase, converts glucose to fructose and develops a mixture of about 50% fructose and 50% glucose syrup. Immobilised GI is used at industrial scale for the production of HFCS[4]. GI also aids in bioethanol production by converting xylose (present in hemicellulosic materials) into xylulose, an easily fermentable sugar[5]. Many microbes are known to produce Glucose isomerase which range from anaerobic *Clostridium thermosulfurogenes, Thermoanaerobacter*[6] and *Thermoanaerobacterium*[7] through aerobic *Pseudomonas, Bacillus* to alkalophillic and acidophilic actinomycetes[1]. Streptomycetes have been reported as one of the preferred category of organisms for production of this enzyme. Streptomycetes are filamentous bacteria which possess extensive genes of primary and secondary metabolism. They are present in soil in abundance and are responsible for the degradation of all kinds of organic matter. This efficiency can be accounted by their capacity to produce different types of enzymes; amylase, protease, lipase, cellulase, glucose isomerase, xylanase, pectinase etc. Production of GI is readily studied from Streptomycetes[8-10].

GI is a thermostable enzyme consisting of four polypeptide subunits of 43 kilo Daltons each. This homotetramer exhibits optimum activity in the presence of magnesium and cobalt ions. Magnesium is reported to be responsible for optimum enzyme activity and cobalt helps in maintaining the structure at elevated temperatures[11,12].

There have been reports about intracellular as well as extracellular production of glucose isomerase[1,5-7]. Majority of the reports are on intracellular production of GI. There is a need of developing a technology for efficient extraction of GI from the microbial cell. In this study we have compared the same among various isolates of *Streptomycetes* and determined an appropriate method for the extraction of intracellular GI.

Material and Methods

A variety of Actinomycetes were isolated from garden soil and compost pit samples. Isolation and purification of cultures was done on Bennett's agar and Actinomycete isolation Agar.

Screening for GI producers: The isolates were screened qualitatively on Xylose and wheat bran media[13,14]. The organisms producing glucose isomerase can isomerise xylose to xylulose besides glucose to fructose. The organisms growing on medium containing xylose as a sole source of carbon will be utilizing xylose as a source of carbon. Xylose has to be first converted to xylulose which is further channelized into pentose phosphate pathway for generation of energy. The organisms possessing very low or negligible GI activity might not grow on such a media. The screening strategy was designed according to the method described by Manhas and Bala[13] with some modifications. We used three different media combinations for primary screening, X+P+ medium, X+P- medium and wheat

bran agar medium. The medium composition of X+P+ and wheat bran agar were according to Manhas and Bala[13] and X+P- was same as X+P+ but excluding peptone. The cultures were spot inoculated on all the media combinations and incubated at 30°C. The isolates developing early on the plates and giving luxurious growth were picked up as GI producers.

Submerged fermentation for GI production: The selected isolates were subjected to submerged fermentation in Bennett's broth. The fermentation period for GI production was optimized. A circular disc of 8mm diameter was cut from the Wheat Bran agar medium as inoculum. They were incubated on orbital shaker Remi C – 24 at 30˚C, 100 RPM for 96 hours. Fermentation process was terminated on fourth day and intracellular and extracellular GI production was examined[1,15]. The growth curve of *Streptomyces SB – AII$_4$* was studied by estimating the increase in biomass at 24 hrs. interval[16]. The experiment was run in triplicates and the average of the readings obtained was used to observe the results.

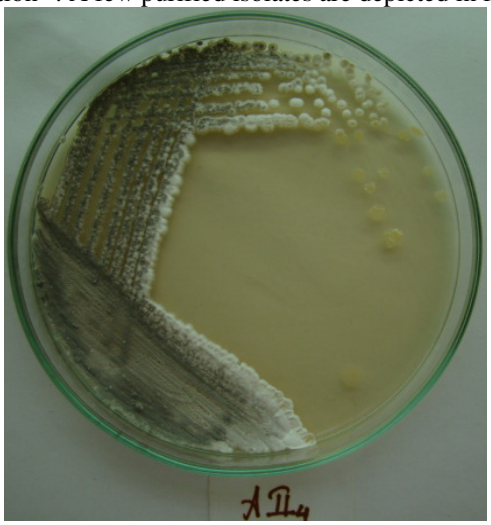
Preparation of enzyme extracts: The broth was harvested and centrifuged at 5000 rpm for 10 mins to get crude extracellular enzyme extract. The centrifuged biomass was washed thrice in sterile distilled water and treated with 0.1% CTAB for 2 hours in shaking condition. It was then centrifuged at 10000 rpm for 10 mins. The supernatant was used as crude intracellular enzyme extract[17].

Glucose Isomerase Assay: Glucose isomerase was assayed by the method described by Chen et. al.[15]. The reaction mixture contained 500µL of 0.2M phosphate buffer, 200µL of 0.1M glucose, 100µL of 0.1M $MgSO_4$ 100µL of 0.01M $CoCl_2$ and 200µL of enzyme extract. The volume was made up to 2mL. The isomerisation was carried out at 70°C for 1 hr. The reaction was stopped by adding 2mL of 0.5M perchloric acid. The amount of fructose formed was estimated by Dische and Barnfoed's method[18]. To an aliquot of 0.05mL of above 0.95mL of distilled water was added. Purple colour developed on adding 200µL of 1.5% Cysteine hydrochloride, 6mL of 70% H_2SO_4 and 200µL of 0.12% Alcoholic Carbazole, was read spectrophotometrically at 560nm[18].

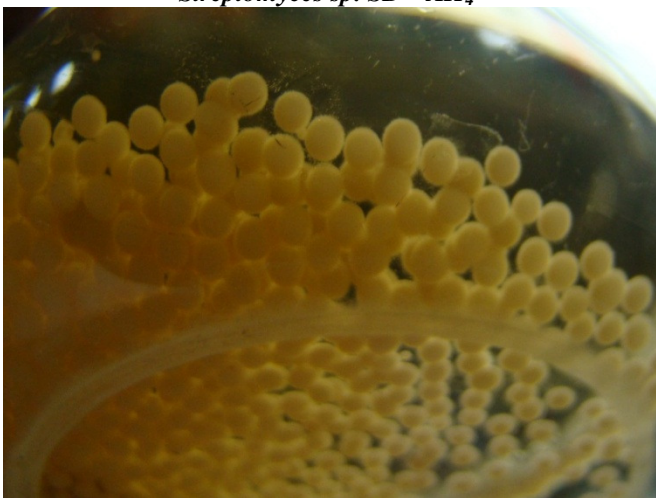
Comparison of Extraction Methods: High GI yielding cultures were checked for both extracellular and intracellular enzyme production. Submerged fermentation was done in Bennett's Broth. The isolate with highest intracellular GI production was selected for extraction studies. A comparison between various physical and chemical methods was done. The biomass was washed and subjected to physical methods like grinding, homogenization and Ultrasonic disruption for 10 mins, 20 mins and 30 mins. Chemical methods included treatment with detergents like CTAB, Tween 80, Triton X100 and SDS[19,20]. Osmotic shock treatment was given by treating the biomass with glycerol and sodium chloride.

Results and Discussion

GI producing isolates: An assortment of varied isolates was developed. The cultures from different sources were categorized into groups like *Streptomyces, Saccharomonospora, Actinomadura, Streptoverticillium, Saccharopolyspora, Nocardiopsis*etc. This differentiation could be achieved by cultural characters and morphological details studied by slide culture technique[21]. The isolates grew as velvety, leathery or chalky colonies on agar plates (figure-1). Streptomycetes being filamentous bacteria grew in liquid medium as beads (figure–2). The isolates exhibited concentric rings around the colonies on ageing which is a typical characteristic of Streptomycetes (figure-3). The samples were picked up from a variety of locations example compost pit, gardens, agricultural area and roadsides. The cultures isolated from these samples were diverse in their spore mass colour, colony reverse and pigmentation[22]. A few purified isolates are depicted in figure–4.



Figure–1
Streptomyces sp. SB – AII$_4$



Figure–2
Growth of *Streptomyces sp. SB – AII$_4$* in liquid medium

Figure–3
Colonies showing concentric rings on Agar Medium

Figure–4
Purified Actinomycete isolates

The preliminary qualitative screening revealed that 36 out of 75 isolates were tentative GI producers. Each isolate responded differently on three media (X+P+ medium, X+P- medium and wheat bran medium). Response of the isolates was best on wheat bran agar medium but their growth was less flourished on X+P+ and X+P- media. The isolates giving early appearance on wheat bran medium also grew well on other two media. X+P+ and X+P- media gave a clear picture of isolates as GI producers, as former is containing xylose, peptone and mineral salts whereas latter is containing only xylose and mineral salts. The growth of organism on X+P- media (in absence of peptone) indicates the production of GI for utilizing xylose present in the media[13]. The isolates which grew well on both X+P+ and X+P- media were picked up as good producers of GI and those growing only on X+P+ were grouped as moderate GI producers. The isolates exhibited scanty growth on X+P- media. This process indicated 36 tentative GI producers with 18 promising ones[23]. A comparison of growth on all the above media is shown in figure - 5. The early appearance of GI producers is depicted in the figure.

The cultures selected by this method were further screened by subjecting to submerged fermentation process. Production of GI was estimated using the selected 36 isolates.

Majority of the cultures exhibited isomerisation ability but 18 of these isolates gave higher yields. The enzyme activity was higher for the isolates which appeared early on the xylose plates. Many isolates were found to produce GI. The enzyme yield ranged from 0.97 Units/mL to 2.8 Units/mL. Different isolates gave varying extent of growth which can be accounted by different medium requirements and growth phases of the isolates. The medium used for primary screening did not contain any inducer as used by some investigators[1,15,24]. The absence of inducer in the fermentation medium was to spot the isolates which do not require the inducer xylose for GI production. As most of the organisms reported by other researchers requires xylose, which increases the production cost. This strategy shall provide an economically feasible process.

Comparison of intracellular and extracellular GI production: A comparative analysis showed that many cultures were good extracellular producers and some were good intracellular producers of glucose isomerase. The promising GI producers were characterized and the organisms were identified by morphological, cultural and biochemical characterization. After repeated comparisons among the isolates *Streptomyces sp. SB – AII$_4$* was found to be the best intracellular glucose isomerase producer and *Streptomyces sp. SB -P$_1$* to be the best extracellular producer (figure - 2). Most of the researchers have reported glucose isomerase as an intracellularly produced enzyme[9,25-31] whereas there are very few reports on extracellular production of GI[13,15,32]. Some of these also reported extracellular as well as intracellular presence of the enzyme.

The isolate being a filamentous bacterium grew as beads in the liquid medium. The beads grew in size and number with increase in incubation time. The progressive increase in biomass was observed till the fifth day but no substantial increase was found on further incubation. Growth of *Streptomyces SB – AII$_4$* is depicted in the form of dry biomass in figure - 7.

Intracellular GI produced by *Streptomyces SB – AII$_4$*could be detected after 24 hrs. of fermentation period. The early appearance of the enzyme must be due to its involvement in primary metabolism. The study shows that GI production increases with growth and reaches to maximum towards the end of exponential phase. The highest enzyme activity was observed in 96 hrs. which is in accordance with results reported by Chou C.C. et. al.[33] and Dhungel B. et. al.[26]. Srih-Belghith K. and Bejar S.[14] reported maximum accumulation of enzyme in 48 hrs. whereas Chen W.P. et. al.[15] and Lobanok A.G. et. al.[34] stated maximum accumulation in 72 hrs. The intracellular and extracellular production of GI by *Streptomyces SB – AII$_4$*is depicted graphically in figure – 8.

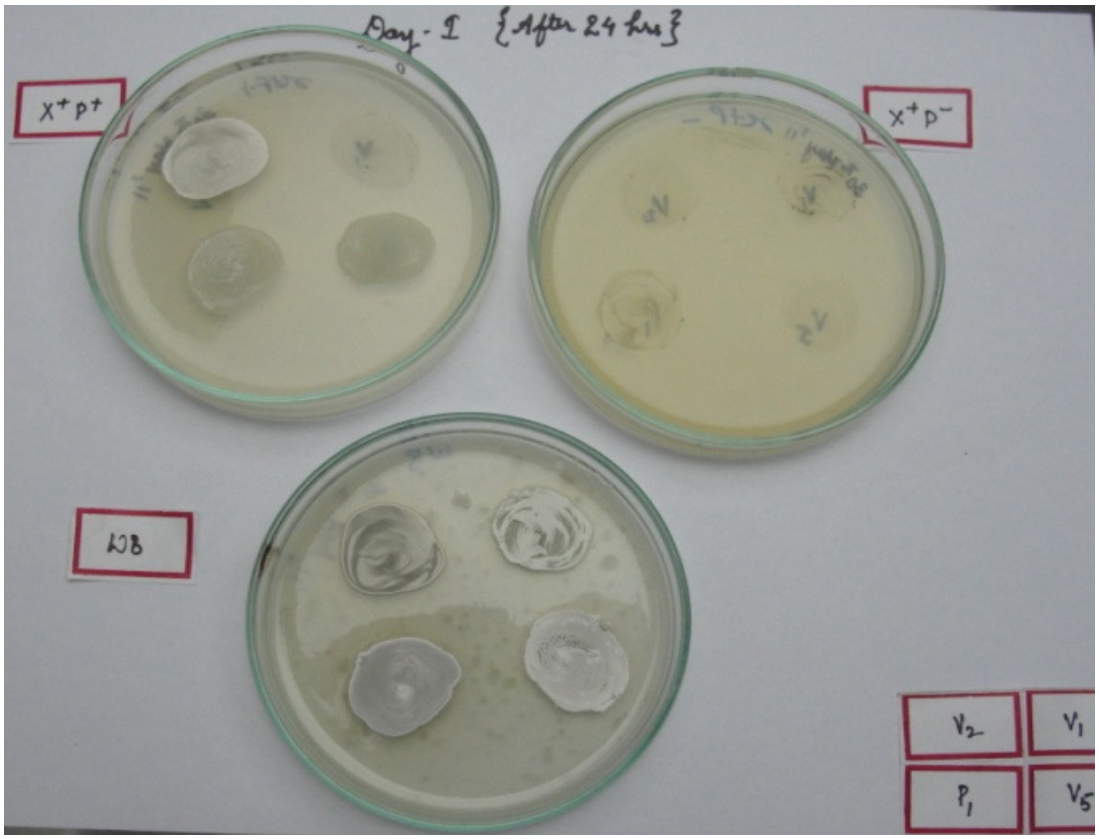
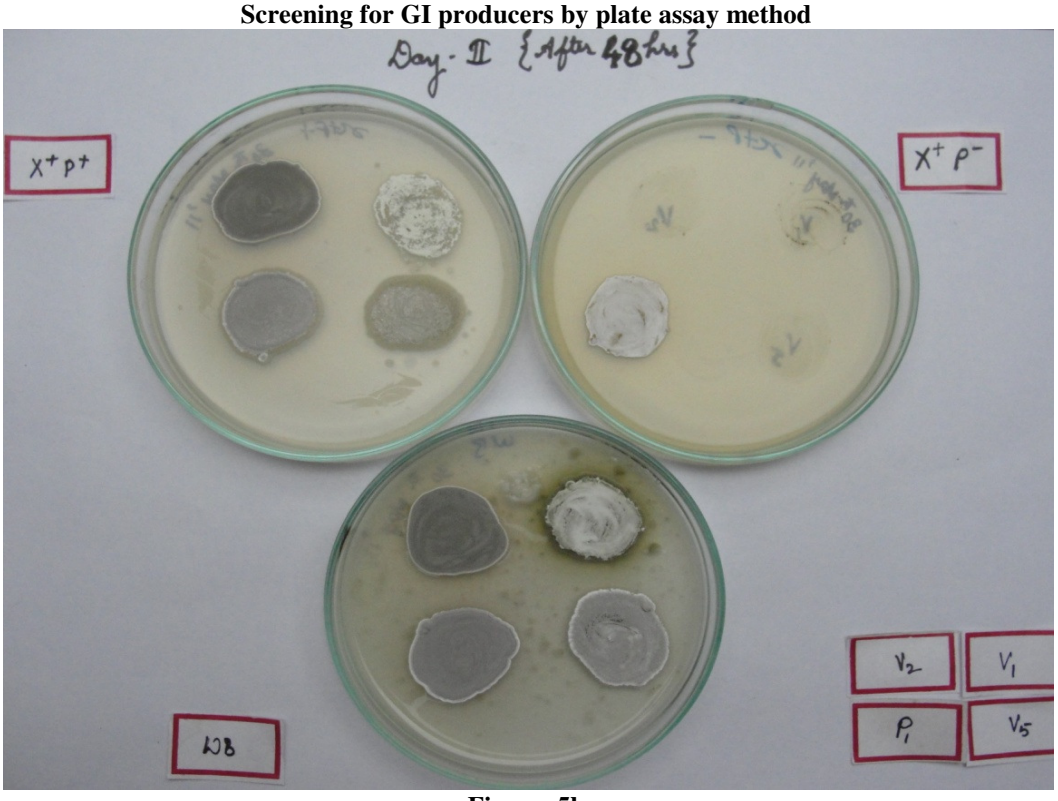


Figure-5a
Screening for GI producers by plate assay method



Figure–5b
Screening for GI producers by plate assay method

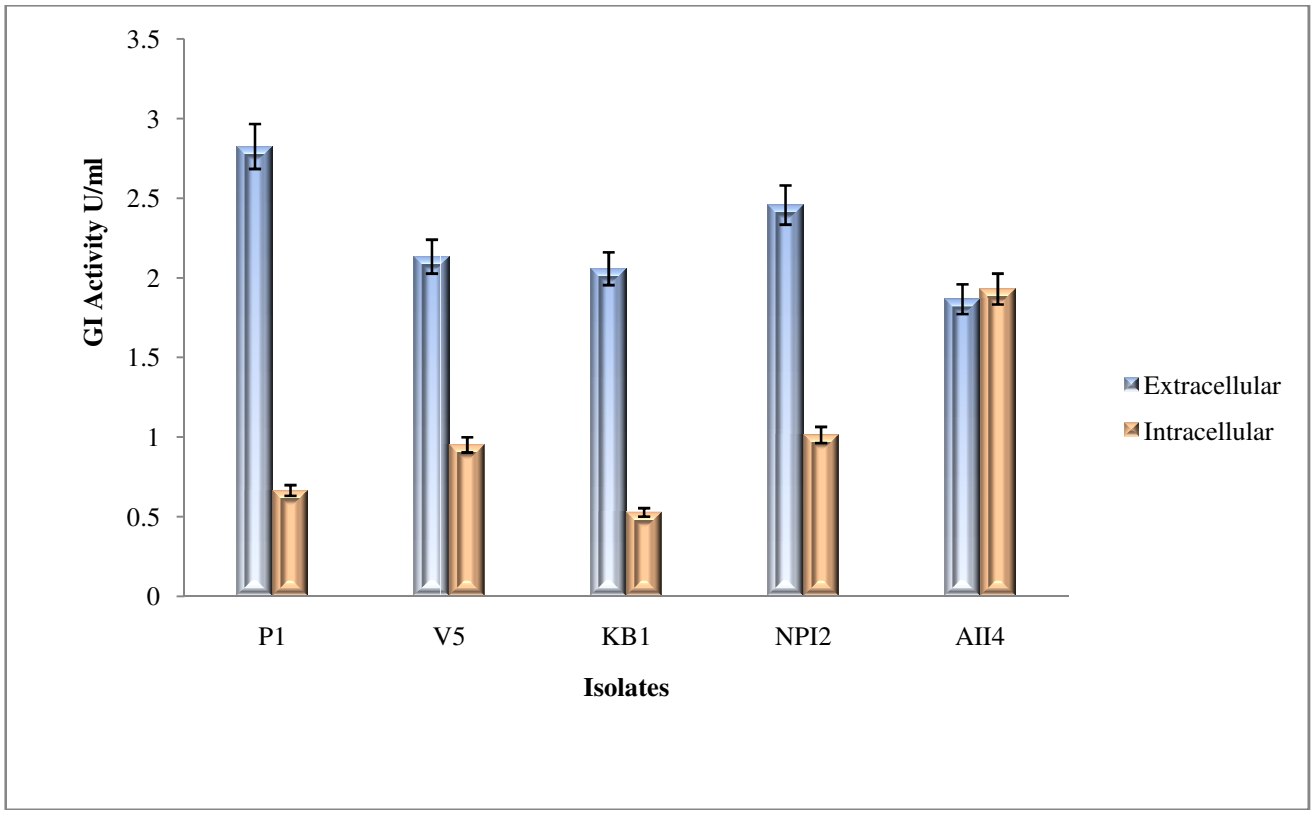


Figure-6
Comparison for best intracellular and extracellular producer of Glucose Isomerase

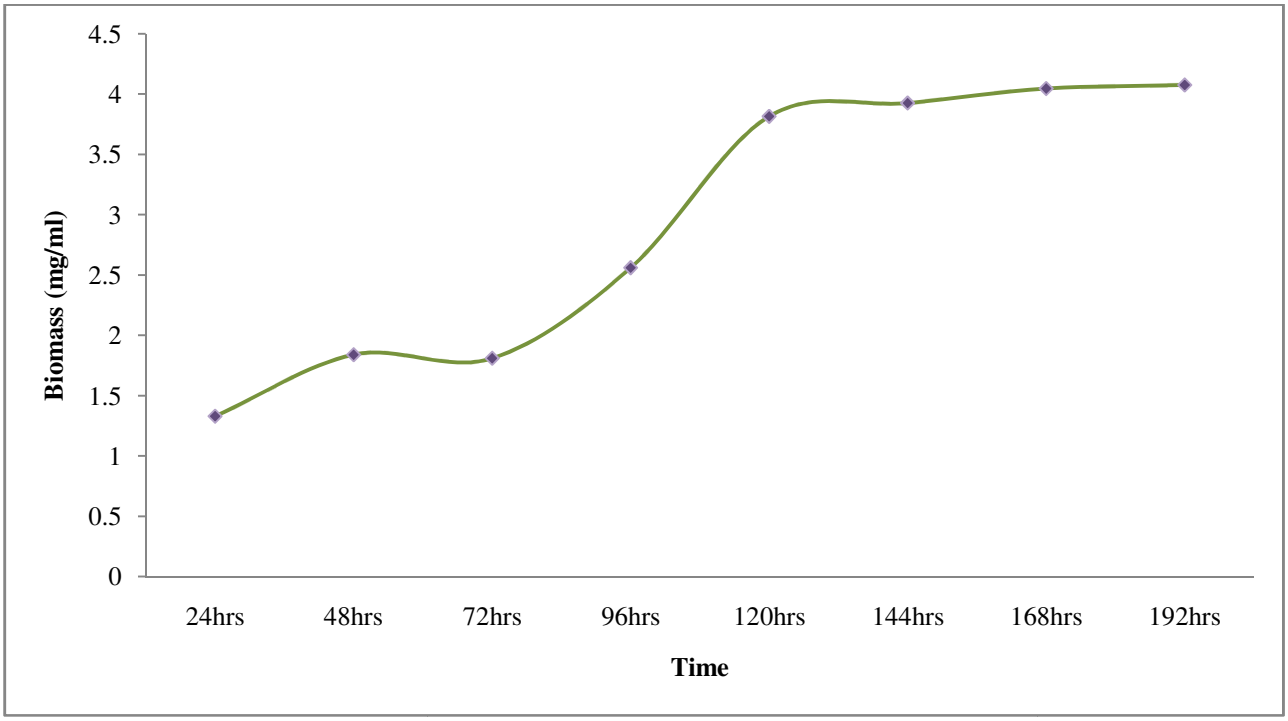


Figure-7
Growth curve of Intracellular GI producer *Streptomyces sp. SB - AII4*

Comparison of Extraction Methods: The downstream processing usually accounts for a considerable cost in the fermentative production process. In this case, where GI is produced intracellularly there should be complete extraction of the enzyme from the microbial cell, in order to get maximum returns of the investment. The comparative analysis indicates ultrasonication to be the best method for the extraction of intracellular enzyme (figure-9). As the treatment time was increased the release of enzyme was also increased. CTAB treatment also yielded good amount of enzyme. Treatment with CTAB[17] and ultrasonication[26,27,35,36] has been a method of choice of many researches because it gives good enzyme yield as compared to other processes.

The biomass was subjected to different disruption methods, for checking the intracellular presence of glucose isomerase. The comparison of activity marked clear difference between the high efficiency of CTAB and Triton to release large amount of enzyme from cells than the performance of NaCl and Glycerol. This also approves CTAB and Triton to be better surface active agents than others, indicating better scope for industrialization of the process.

Gong et al 1980[35] reported the extraction of GI from *Actinoplanes missouriensis* and Azin et al. 1997[9] from *Streptomyces olivochromogens*, Dhungel et al. 2007[26] also extracted thermostable GI from *Streptomyces sp.* and Lama et al. 2001[27] from *Bacillus thermoantarticus* by sonication. Demnerova et al. 1982[36] compared methods for extraction of GI from *Streptomyces nigrificans* and observed maximum yield by autolysis with toluene and lysozyme. They found that Novotony's disintegration method was best among all the physical methods.

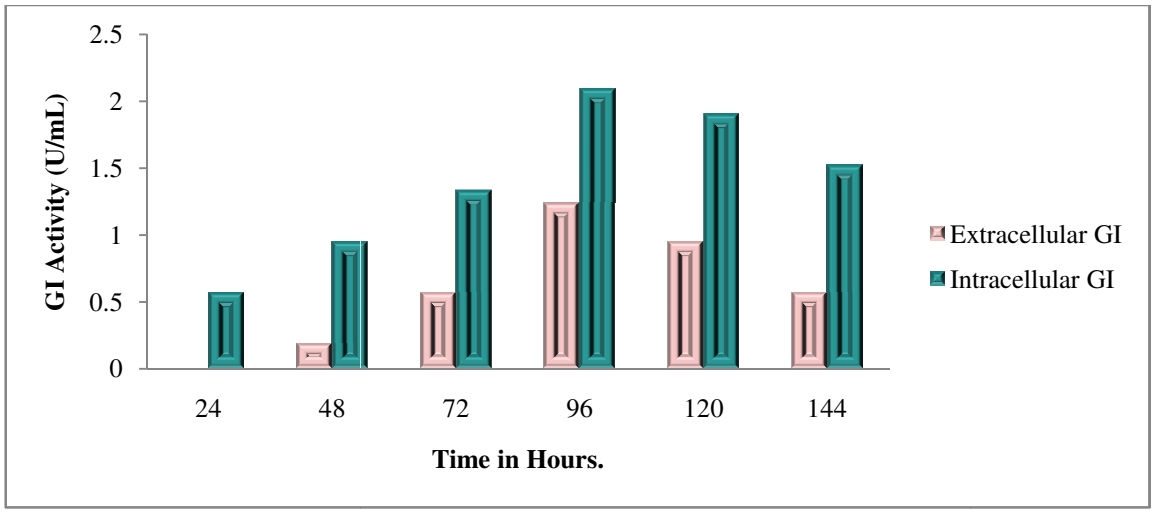


Figure-8
Optimization of incubation period for GI production by AII_4

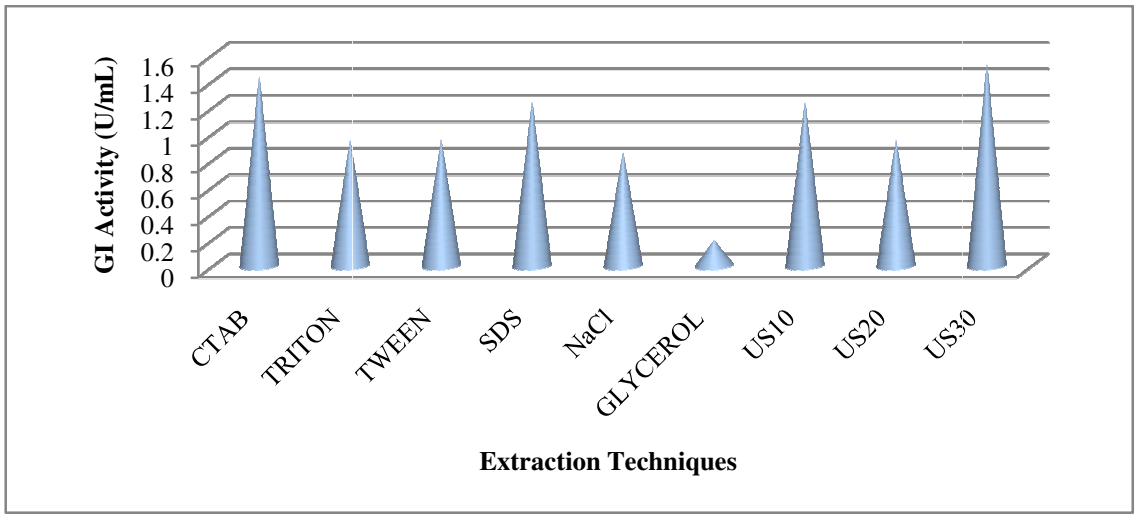


Figure-9
Comparison of intracellular enzyme extraction methods for *Streptomyces sp. SB – AII_4*

Conclusion

The study has pointed out the presence of intracellular as well as extracellular producers of GI among the microbes isolated from soil of Indore, MP, India region. The recovery of maximum amount of enzyme produced by the organism can be done only by using an efficient disruption technique. Ultrasonication and treatment with CTAB proved to be the best methods for extraction of the GI. High recovery of GI by ultrasonication is also reported by other researchers[27,36]. Detergents like SDS and CTAB extracted good amount of enzyme from the cells. Ultrasonication is an efficient method for handling less volumes of fermentation medium especially for research purposes whereas for industrial applications high recovery can be achieved by economic processes like treatment with surfactants such as CTAB. The organism is also releasing GI extracellularly in the fermented broth which can be detected from the early stages of growth phase. The release of enzyme in the medium cannot be overlooked and should be recovered in the downstream processing. Presence of extracellular GI is very less in the early stages of growth but with increase in fermentation period the amount of enzyme released is also increasing which is in significant amount. The release must be due to the autolysis of cells at this stage of incubation. Summing up the extracellularly released GI with the intracellular GI extracted by an efficient method can increase the viability of the process.

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