Production and application of glucose isomerase from *Streptomyces enissocaesilis* and amylase from *Streptomyces* sp. for the synthesis of high fructose corn syrup

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Abstract

This study reports *Streptomyces* sp. strain T.S.A.KP identified as *Streptomyces enissocaesilis* isolated from soil inhabiting actinobacteria of Madhya Pradesh (India) as a new actinobacterial strain for production of glucose isomerase (GI) and *Streptomyces* sp. strain TSA. Dm1 closely related to *Streptomyces sampsonii* for the production of amylase. These strains were selected from an array of 120 actinobacterial isolates, which on screening originally yielded 18% GI producers and 36% amylase producers. *S. enissocaesilis* yielded maximum GI at pH 7 and 70 °C in 120 h. Highest amylase production from *Streptomyces* sp. strain TSA. Dm1 was observed at pH 7 and 37 °C in 96 h. Michaelis–Menten constant (K_m) for GI and amylase was 1.54 M and 0.36 M, respectively. V_{max} for GI was 11.76 U/ml and amylase 33 U/ml. These cultures were employed for HFCS production. Saccharification of corn starch was achieved up to 90.9% by *Streptomyces* sp. strain TSA. Dm1, and the glucose was isomerized to 40.8% by *S. enissocaesilis*. Presence of glucose and fructose in HFCS was also confirmed by high-performance liquid chromatography.

Keywords Actinobacteria · *Streptomyces* · Amylase · Glucose isomerase · High fructose corn syrup

1 Introduction

High fructose corn syrups (HFCS) in the past half century have gained remarkable attention as a sweetener for food and beverage industry. This liquid sweetener was developed in the 1960s as a partial replacement to sucrose which suffered production shortages and price hikes after Cuban revolution [1]. HFCS is an equilibrium mixture of glucose and fructose which has sweetening ability comparable with sucrose [2]. Fructose in HFCS is 1.3 times sweeter than sucrose and 1.7 times sweeter than glucose. Thus, HFCS possesses a higher sweetening capacity. This sweetener possesses a high commercial value, and its annual production is estimated to be 10 million tons [1].

Fructose is twice as sweet as sucrose, and glucose imparts 70–75% sweetening as that of sucrose [3]. HFCS

is chiefly produced from non-sweet sources like starch or cellulose which makes it cheaper than sucrose [4]. Apart from this, HFCS is stable and does not pose the problem of crystallization. Its commercial manufacturing began from 1970s opening a new frontier in the sweetening market.

The industrial production of HFCS requires two important stages. In initial step, amylase is employed for saccharification of non-sweet starch to monomeric glucose units followed by its isomerization into fructose using glucose isomerase [5]. Usually two variants, HFCS-42 (42% fructose) and HFCS-55 (55% fructose) are available commercially [6]. The concentration of fructose in HFCS can be enriched to 90%, but industrially HFCS-55 is chiefly used as sweetener in cold drinks, ice cream and yogurts. HFCS-42 finds its usage in confectionary and beverage industries [7].

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The HFCS is produced globally, 41% of which is consumed by the beverage industries of USA. In India, sugarcane-derived sugar is still the chief sweetener. These sweeteners need to be replaced with HFCS as it has numerous benefits. HFCS is useful for diabetic patients also. It has low calorific value, and moreover insulin is not required for absorption of fructose in blood. HFCS can be transported easily in its liquid form as there is no problem of crystallization [8]. Due to its medical advantages, HFCS production on large scale from cheap starchy source is in demand. Owing to the great abundance of starchy by-products in India and high international market demand of HFCS, we can establish plants here to earn foreign exchange which will increase our share in international trade.

In this study, potential GI- and amylase-producing actinobacteria have been screened and identified using morphological and molecular techniques. The 16S rRNA sequence of both the selected strains *Streptomyces* sp. TSA.KP (KP) and *Streptomyces* sp. TSA.Dm1 (Dm1) has been submitted to NCBI, and this is the first report on utilizing these organisms for the production of GI and amylase, respectively. Different production media were checked for maximum production of GI and amylase, and properties of both the enzymes were studied. The enzymes produced were later used for the production of HFCS.

2 Materials and methods

All the chemicals used were purchased from HiMedia and Merck.

2.1 Isolation of actinobacteria from soil

The soil samples from forty different regions of Madhya Pradesh, India, were collected in sterile polybags. Soil samples were procured from 5 to 6 cm depth using a spatula. The isolation was carried out using actinomycetes isolation agar and Bennett's agar medium. Selective isolation of actinobacteria was promoted by incorporating amphotericin B (20 µg/ml), ampicillin (3 µg/ml) and erythromycin (1 µg/ml). Morphologically diverse actinobacteria were procured and preserved on Bennett's agar slants for further studies.

2.2 Screening for production of glucose isomerase (GI)

The actinobacterial isolates were spot inoculated on X + P[−] (xylose 1%, KNO₃ 0.2%, K₂HPO₄ 0.2%, NaCl 0.2%, MgSO₄ 0.05%, FeSO₄ 0.01%, CaCO₃ 0.02% and pH 8.0 ± 0.2), X + P⁺ and wheat bran medium for primary screening [9] and incubated at 30 °C for 7 days. The X + P⁺ media is comprised peptone (0.03% w/v) additionally along with all the

other components of X + P[−] [9]. The plates were observed regularly for growth. The isolates exhibiting early lavish growth on X + P[−] media were considered good producers of GI. GI is considered to be intracellular in majority of the cases; in this study, we intend to focus on extracellular GI production in order to reduce the product recovery cost. Therefore, in secondary screening we performed a comparative study of extracellular and intracellular GI production.

The submerged fermentation process was carried out in modified Bennett's medium (xylose 1%, tryptone 0.1%, yeast extract 0.1%, beef extract 0.1% and pH 8.0 ± 0.2). Top ten promising GI producing isolates were inoculated using 8-mm culture disk of 96-h-old culture of isolates. The inoculated broth was incubated in a rotatory shaker (REMI CIS-24BL) at 30 °C at 100 rpm. Fermentation process was terminated after 96 h, and the fermented broth was centrifuged at 5600×g for 10 min. The supernatant was used as crude extracellular enzyme extract.

To estimate the production of intracellular GI, the pellet obtained after centrifugation in the above procedure was washed thrice using distilled water (pH 7.0). Further, it was suspended in 0.05 M sodium phosphate buffer (pH 7.0) containing 0.1% cetyl trimethyl ammonium bromide (CTAB). This mixture was incubated in shaking condition at 37 °C for 24 h at 100 rpm. Later, it was centrifuged at 5600×g for 5 min and the supernatant was used as crude intracellular enzyme [11].

2.3 Screening for production of amylase

The isolates were tested for synthesis of amylase using plate assay method. The isolates were spot inoculated on Bennett's medium containing 1% starch and incubated at 30 °C for 4 days. Plates were flooded with iodine solution to observe the zone of hydrolysis [10].

The isolates exhibiting large zone of clearances in the plate assay method were analyzed further for amylase production by submerged fermentation process. The potential isolates were inoculated into Bennett's medium containing 1% starch and incubated at 30 °C for 4 days at 100 rpm. The fermented broth was harvested after incubation and assayed for amylase production.

2.4 Selection of suitable production medium for GI

Production of GI is greatly influenced by the nutrient combination present in the medium; in order to enhance the GI production, different media were tested. GI was produced in Bennett's medium, modified Bennett's medium, Lobanok medium [12], Srih Belghith medium [13], Hasal medium [14] and X + P⁺ [9] production media by submerged fermentation process for selection of suitable

production medium. The GI produced was estimated by the method suggested by Chen et al. [18].

2.5 Selection of suitable production medium for amylase

Top three amylase producers were subjected to submerged fermentation process in four different production media: Haritha's medium [15], Acharyabhatta medium [16], Raghunathan medium [17] and Bennett's medium for determining the most suitable production medium. The amylase produced was estimated by the method suggested by Raghunathan and Padhmadas [17].

2.6 GI assay

The GI was assayed by preparing a reaction mixture containing 500 μ l of 0.5 M phosphate buffer, 200 μ l of 1 M glucose, 100 μ l of 0.1 M MgSO_4 , 100 μ l of 0.01 M CoCl_2 and 200 μ l of crude enzyme. The volume was made up to 2 ml, and the reaction was carried out at 70 °C for 1 h. The reaction was stopped using 2 ml 0.5 M perchloric acid [18].

To estimate the amount of fructose formed, 0.05 ml of above aliquot was taken to which 0.95 ml D/W was added followed by 200 μ l of 1.5% cysteine hydrochloride, 6 ml 70% H_2SO_4 and 200 μ l of 0.12% alcoholic carbazole. The purple color thus formed was read spectrophotometrically at 560 nm [19], and the concentration was determined using fructose calibration curve. One unit of glucose isomerase activity was defined as the amount of enzyme required to produce 1 μ mol of fructose per minute, under assay conditions.

2.7 Amylase assay

The amylase was assayed by adding 0.2 ml of enzyme extract to 0.5 ml 1% starch solution. The reaction mixture was incubated at 37 °C for 30 min. The reaction was stopped by adding 1 ml DNS to the reaction mixture. The reaction mixture was kept in boiling water bath for 10 min, and the total volume was made to 5 ml. The absorbance was read at 540 nm using UV-visible spectrophotometer (ThermoSpectronic).

One unit of amylase enzyme activity was defined as the amount of enzyme required to liberate 1 μ mol of glucose per min under the assay conditions [17].

2.8 Identification of GI-producing isolate KP and amylase-producing isolate Dm1

The identification of the GI- and amylase-producing actinobacteria was carried out in two steps, i.e., morphological analysis and molecular characterization.

2.8.1 Morphological analysis of isolate KP and Dm1

The selected GI and amylase producers were analyzed for their morphological details by studying spore arrangement pattern. Preliminary morphological analysis of selected isolates was performed by slide culture technique. Detailed morphological analysis was done by scanning electron microscopy at 18000X and 19000X.

2.8.2 Molecular characterization of isolate KP and Dm1

Molecular characterization of isolate KP and Dm1 was performed by 16S rRNA sequencing. The chromosomal DNA of both actinobacteria was isolated using the DNA isolation spin column kit (HiMedia). The 16S rRNA was amplified using 27F (5'-GAGTTTGATCMTGGCTCAG-3') as the forward primer and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') as the reverse primer [20]. The PCR product was purified using Exonuclease 1—Shrimp Alkaline phosphatase (Exo-Sap), and then the amplicons were sequenced through Sanger's method using a ABI 3500xL genetic analyzer (Life Technologies, USA). This process of identification of actinobacterial species was carried out at National Collection of Industrial Microorganisms (NCIM), NCL-CSIR, Pune, India. The sequence obtained was matched with the GenBank database using BLASTN algorithm to reveal the closest match of 16S rRNA from the known species. Phylogenetic tree was constructed using sequence of our isolate and the first 25 closely related sequences picked up from BLAST result using MEGA-X program.

2.9 Determination of optimum fermentation period for GI production and study of growth pattern of isolate KP

This study was done by inoculating 8-mm culture disk (from 9-h-old culture of isolate KP) into ten flasks containing modified Bennett's medium. Submerged fermentation process was carried out at 30 °C in shaking condition at 100 rpm. Daily, a flask was drawn, its contents were centrifuged at 5600 $\times g$ for 10 min, and the supernatant was assayed for GI activity. The growth pattern of the isolate was studied by estimating the increase in biomass. During the time course of 10 days, biomass was estimated by measuring the dry weight of the growth obtained at an interval of 24 h. The pellet was washed with distilled water and was filtered through preweighed Whatman's filter paper and dried overnight at 45 °C. After complete drying, the filter paper was weighed again and biomass was recorded.

2.10 Determination of optimum fermentation period for amylase production and growth pattern of isolate Dm1

To determine the optimum incubation period for maximum amylase production, 8-mm culture disk from 96-h-old culture of isolate Dm1 was inoculated into ten flasks containing Haritha's medium. Submerged fermentation was performed at 30 °C at 100 rpm. Each day a flask was drawn, the fermented broth was centrifuged at 5600×g for 10 min and the supernatant was assayed for amylase. Growth was estimated by analyzing the dry weight [17]. The pellet was washed with distilled water and was filtered through preweighed Whatman's filter paper and dried for overnight at 45 °C. The filter paper was weighed again after complete drying, and the biomass was recorded.

2.11 Effect of temperature on GI and amylase activity

The effect of temperature on the activity of both GI and amylase was investigated. For determination of optimum temperature for GI activity, the isomerization process was carried out at different temperatures, i.e., 50 °C, 60 °C, 70 °C, 80 °C, 90 °C and 100 °C. Similarly, an optimum temperature for amylase activity was determined by carrying out the starch hydrolysis at different temperatures 15 °C, 22 °C, 30 °C, 37 °C, 42 °C and 55 °C.

2.12 Effect of pH on GI and amylase activity

The effect of pH on GI and amylase activity was determined by carrying out isomerization and hydrolysis reactions, respectively, at different pH ranges from 4 to 12. Different buffers were used for maintaining constant pH of the reaction system. For pH 4 and 5, acetate buffer was used, phosphate buffer for pH 6 and 7, tris buffer for pH 8 and 9, and glycine buffer for pH 10, 11 and 12.

2.13 Kinetic properties of GI and amylase

Kinetic properties of GI and amylase were determined using Lineweaver–Burk plot. Glucose was used as the substrate for studying kinetic properties of GI, and similarly starch was employed as the substrate for analyzing kinetic properties of amylase. Both the substrates used in this study were introduced in the concentration ranging from 0.1 to 2.0 M. The pH for both GI and amylase for kinetic studies was 7.0, and temperature was kept 70 °C and 37 °C, respectively.

2.14 Production of HFCS

Corn starch was utilized as the initial polysaccharide, and the process of HFCS production was done in two steps. The initial process involved hydrolysis of corn starch into glucose subunits for which the crude amylase from isolate Dm1 was utilized. The corn starch (1% w/v) was dissolved in 0.5 M phosphate buffer (pH 7.0) to which crude enzyme was added and incubated at 37 °C. The mixture was tested at different time intervals for decrease in level of starch which was estimated using starch iodine complex method. The amount of glucose units generated was estimated using the DNS method. This step was followed by isomerization of glucose units into fructose which was done by adding crude GI from isolate KP to the above mixture. Isomerization was carried out at 70 °C. The reaction mixture was tested for the production of fructose until no rise in isomerization was observed. The amount of fructose produced was estimated using the cysteine-carbazole method.

2.15 End product analysis using HPLC

The HFCS produced using amylase and GI from *Streptomyces sp.* was analyzed for the presence of sugars. This analysis was done using HPLC, Waters, USA, equipped with Sugar-Pak column of 6.5 × 300 mm, 515 HPLC pump and RI detector 2414. The injection volume was 20 µl, and HPLC-grade water was used as mobile phase with flow rate of 0.3 ml/min. The HFCS produced was filtered using PVDF syringe equipped with a 0.45 µm filter, and the filtrate was injected (20 µl) into the HPLC system using Hamilton's syringe. The analysis was carried out using Empower 2 software.

3 Results and discussion

3.1 Isolation of actinobacteria from soil

Soil inhabits diverse groups of microbiota which can serve as a resource for the isolation of microorganisms with desired properties. Actinobacteria cover a major portion of microbial soil population and are considered valuable due to their ability to produce numerous enzymes for mineralization and diverse secondary metabolites. In our present study, soil samples gathered from 40 locations yielded 120 morphologically different actinobacterial isolates which were preserved on Bennett's agar slants. The sampling area included soil from garden, cultivated land, compost pits, rhizospheric soil, wastelands and dump yards. Actinobacteria were found to be present in all the samples collected. From the 12 garden soil samples, 43

Fig. 1 Isolation of actinobacteria from different soil samples

actinobacterial cultures were isolated and rhizospheric soil yielded 28 actinobacterial isolates followed by that of compost pits (18 isolates), cultivated land (17 isolates) and wastelands (14 isolates). The presence of organic matter and other nutritional sources might be the reason for this observation. The details of the isolation are mentioned in Fig. 1.

3.2 Screening for production of glucose isomerase (GI)

All actinobacterial isolates were checked for their ability to produce GI. Results exhibited that among 120 isolates, only 25 exhibited GI activity which accounts to 18% of the total. The ability of glucose isomerase to convert xylose into xylulose was utilized for the screening purpose. The microbes cannot utilize xylose directly as the source of carbon but instead convert it into xylulose for consumption. X + P⁻ medium comprises solely of xylose as the source of carbon allowing the selective growth and survival of GI producing isolates. The isolates exhibiting early lavish growth on this medium were considered good producers of GI. On the contrary, X + P⁺ promotes better growth as it contains peptone, whereas wheat bran facilitates early sporulation as it is rich in growth factors. Wheat bran and X + P⁺ media were used as positive controls. As a result of primary screening, 4 isolates were selected for secondary screening. Glucose isomerase has been reported in majority of cases as an intracellular enzyme. The processing and isolation of such enzymes make it expensive due to which extracellular GI has been of particular interest. In our investigation, we analyzed both intracellular and extracellular production and the isolates BD1, BD2, KP and S3 were found to be the best GI producers. These selected isolates produced extracellular as well as intracellular GI (Table 1).

Table 1 Comparative analysis of extracellular and intracellular GI production exhibited by different isolates in modified Bennett's medium

Isolate code	Modified Bennett's medium	
	Extracellular	Intracellular
RK1	2.40 ± 0.38	1.36 ± 0.62
BD1	3.36 ± 0.63	1.71 ± 0.45
BD2	3.26 ± 0.51	0.58 ± 0.28
KP	4.72 ± 0.78	1.93 ± 0.43
HO6	2.94 ± 0.33	1.06 ± 0.26
OM2	1.33 ± 0.38	0.18 ± 0.09
PS2	2.29 ± 0.47	0.85 ± 0.31
CK	2.67 ± 0.73	1.12 ± 0.53
Db1	1.49 ± 0.52	0.64 ± 0.24
S3	4.27 ± 1.03	0.90 ± 0.37

The values refer to enzyme activity in EU/ml (mean ± SD, *n* = 3)

The isolate KP exhibited the highest extracellular enzyme production in modified Bennett's medium. Majority of isolates exhibited more extracellular enzyme accumulation as compared to intracellular. Here we focus on the production of extracellular GI as they are generally stable and easier to purify which reduces the cost of production [21]. In order to achieve maximum recovery of enzyme, both extracellular and intracellular enzymes must be extracted, but at industrial scale it becomes expensive and time consuming. Recovery of intracellular enzymes requires disruption of cell biomass using chemicals or sonicator which increases the overall cost of enzyme production. Thus, extracellular enzymes are of major preference at industrial levels.

3.3 Screening for production of amylase

From the gathered lot, 36% (43 out of 120) isolates exhibited amylase production. Amylase being an inducible enzyme is synthesized by the isolate to utilize the starch incorporated within the medium. The potential of isolate to produce amylase was analyzed by the plate assay method by measuring the zone of clearance around its colony on starch agar plate. The zone diameter of amylase producing isolates ranged from 3 mm to 32 mm (Fig. 2).

The quantitative analysis of amylase production using submerged fermentation process was carried out using eight potential actinobacterial isolates selected from the plate assay method. The potential amylase producing isolates were found to be Dm1, B and S9 which exhibited high amylase production in the submerged fermentation process. The results are shown in Fig. 3.

Fig. 2 Comparative analysis of amylase production by different isolates

Fig. 3 Submerged fermentation process for quantitative analysis of amylase

3.4 Selection of suitable production medium for GI

The growth and production of enzymes and metabolites of actinobacteria depends on the presence of carbon and nitrogen sources in any medium. We analyzed the GI production by top 4 isolates in different medium combinations.

Among the 4 isolates, KP produced maximum amount of GI. The modified Bennett's medium proved to be the best for GI production for actinobacterial isolate KP followed by Lobanok's, and Srih Belghith medium (Fig. 4). In the modified Bennett's medium, glucose has been replaced with xylose which proves that xylose acts as an inducer for GI production as majority of isolates exhibited high GI production in this medium. On the contrary, isolate BD2 exhibited good GI production in Bennett's medium

Fig. 4 GI production in different media

containing glucose as the carbon source. The GI production by isolate BD2 decreased in the presence of xylose, which shows that BD2 utilizes glucose as an inducer for GI production. Xylose is known to induce GI in numerous actinobacteria [1], but there are reports on GI production without xylose as in *Actinoplanes* [22]. Manhas and Bala [21] also reported high GI production in medium containing xylose as the carbon source.

3.5 Selection of suitable production medium for amylase

The selected amylase producers were introduced into different production media. Isolate Dm1 exhibited the highest amylase yield of 88.31 ± 1.31 EU/ml in Haritha's medium followed by isolate S9 (67 ± 1.41) and B (61.1 ± 1.39). Among different production media, Haritha's medium proved to be the most suitable for the production of amylase (Fig. 5). The isolate Dm1 and Haritha's medium promoted the highest amylase production.

3.6 Identification of GI-producing isolate KP and amylase-producing isolate Dm1

3.6.1 Morphological analysis of isolate KP and Dm1

The isolate KP grows well on Bennett's agar medium at 30 °C pH 7 as powdery colonies with gray-colored aerial spore mass, grayish color colony reverse and no pigmentation. Isolate Dm1 also grows on the same medium and with the same physiological parameters as greenish ivory-colored colonies with pale yellow colony reverse and no pigmentation. The spore ornamentation was studied by performing slide culture technique. The aerial mycelia appeared dark in color on thin slender substrate mycelia

Fig. 5 Amylase production in different media

in phase contrast microscope. The spores could be seen as beads in curved arrangement. The scanning electron microscopic pictures revealed spores as small rod-shaped structures arranged in either curved or in spiral chains. The surface of spores appeared rough and wavy for isolate KP (Fig. 6a). The spore chains of Dm1 appeared spiral and the spore surfaces were rough (Fig. 6b). The spore ornamentation is indicative of the isolates belonging to *Streptomyces* sp.

3.6.2 Molecular characterization of isolate KP and Dm1

The complete 16S rRNA sequence of isolates KP (1328 bp) and Dm1 (1214 bp) was obtained. These isolates were identified as *Streptomyces* sp. The sequences were submitted to NCBI. The GI producer is registered in GenBank as *Streptomyces* sp. strain T.S.A.KP accession number MN911386. This strain was identified as *Streptomyces enissocaesilis* on performing BLASTn. This is the first report on *S. enissocaesilis* for the production of GI to the best of our knowledge. Researchers have reported its potential for the production of antimicrobial used in medical as well as agricultural field, but there are no findings available for GI production from it [23, 24]. The amylase producer *Streptomyces* sp. strain TSA.Dm1 is registered in GenBank with accession number MN923073. This isolate was found closely related to *Streptomyces sampsonii*. The phylogenetic tree was constructed using MEGA X program for both the isolates *Streptomyces* sp. strain T.S.A.KP and *Streptomyces* sp. strain TSA.Dm1 separately. The tree was constructed with 26 sequences; i.e. our isolate 16S rRNA sequence and first 25 closely related sequences received from BLAST result. The results revealed query coverage of 99% in BLAST with the score 2438 showing 100% identity with *Streptomyces enissocaesilis* strain NRRL B-16365. The

Fig. 6 **a** Spore chain arrangement of isolate KP under scanning electron microscope. **b** Spore chain arrangement of isolate Dm1 under scanning electron microscope

phylogenetic tree in Fig. 7 also explains the placement of our newly reported culture closest to *Streptomyces enissocaesilis*. BLAST results for *Streptomyces* sp. strain TSA.Dm1 exhibited 100% query coverage with 2377 score and 99.17% identity with *Streptomyces sampsonii* strain ATCC 25495. Tree constructed using these results is depicted in Fig. 8 support that our isolate is clustered in *Streptomyces* group.

3.7 Optimum fermentation period for GI production and study of growth pattern of isolate KP

The highest enzyme production was observed on the 5th day (120 h) from incubation. The study also showed that the production of enzyme increases with respect to the growth of the isolate and attains maxima during the end of logarithmic phase. It is evident in the present study that the enzyme production is taking place in log phase which proves that GI is important for the growth of organism [18,

Fig. 7 Phylogenetic tree of the 16S rRNA gene sequence of *Streptomyces* sp. TSA.KP and other closely related gene sequences

[25]. Different researchers have reported maximum enzyme accumulation between 36 and 96 h [9]. Dhungel et al. [25] obtained maximum production of GI after 96 h (Fig. 9a), whereas Chen et al. [18] achieved the same in 72 h.

3.8 Optimum fermentation period for amylase production and growth pattern of isolate Dm1

The considerable amylase production was observed after 24 h of fermentation process which reached maximum on 96 h (4th day) from incubation (Fig. 9b). The current study establishes that amylase is an important enzyme for the growth of the organism as it is synthesized during the log phase of an organism. Salahuddin et al. [26] also noticed maximum production in 96 h from thermophilic strains of actinobacteria. Some researchers reported reduction

in enzyme activity on 4th day in case of *Rhodococcus* sp. [17], whereas Selvan et al. [27] found the highest enzyme accumulation on 7th day from marine actinobacteria.

3.9 Effect of temperature on GI and amylase activity

The optimum temperature for GI activity was found to be 70 °C, and that of amylase activity was 37 °C. A maximum GI activity of 4.78 ± 0.13 EU/ml was obtained at 70 °C (Fig. 10a). Earlier reports also suggest that isomerization requires a higher temperature range. The isomerization temperature of commercially available GI is 55–60 °C. GI that can carry out isomerization at higher temperatures than the currently available ones enhances the possibility of production of HFCS with higher D-fructose content

Fig. 8 Phylogenetic tree of the 16S rRNA gene sequence of *Streptomyces* sp. TSA.Dm1 and other closely related gene sequences

[28]. For industrial use, GI active at higher temperature is advantageous as it helps in the reduction of viscosity of the substrate.

In the present study, the maximum amylase activity of 88.24 ± 1.32 EU/ml was observed at 37 °C (Fig. 10b). Amylases working at different temperatures have been reported. Kaneko et al. [29] reported *Streptomyces* sp. synthesizing amylase that was able to carry out maximum activity at 40 °C. Yassien and Asfour [30] reported amylase from *Streptomyces clavifer* exhibiting maximum activity at the range of 40–50 °C. Industrially enzyme working at a moderate temperature of 35–40 °C is advantageous as this temperature range can be economically maintained [31].

3.10 Effect of pH on GI and amylase activity

The optimum pH for both GI and amylase was found to be 7.0 (Fig. 11a, b). The GI was found to be considerably

active at the pH range of 6–8. Kovalenko et al. [32] also reported the optimum pH for GI activity from *Actinoplanes missouriensis* to be 7.0. Earlier studies have reported that amylase from *Streptomyces* sp. is active between pH of 6 and 7 [29]. Naragani et al. [33] reported maximum amylase activity from *Streptomyces cheonanensis* at pH 7.0. Singh et al. [34] also reported the optimum pH range of 6–7 for amylase activity of *Streptomyces* sp. MSC701.

3.11 Kinetic properties of GI and amylase

The Michaelis–Menten constant (K_m) for GI and amylase was found to be 1.54 Mol and 0.36 Mol, respectively. The Lineweaver–Burk plot showed that the maximum velocity (V_{max}) for GI and amylase is 11.76 U/ml and 33 U/ml, respectively (Fig. 12a, b). It is difficult to compare kinetic values of reported by other researchers, as they used different concentration of substrate and different assay

Fig. 9 **a** Optimum fermentation period for GI production and growth pattern of isolate KP. **b** Optimum fermentation period for amylase production and growth pattern of isolate DM1

conditions. Fan et al. [35] reported K_m and V_{max} values for GI to be 11 mM and 25 U/mg, respectively. The relatively low value of K_m for both the biocatalysts in the present study demonstrates higher affinity of enzyme for the substrate. Singh et al. [34] observed K_m and V_{max} values of amylase produced by *Streptomyces* sp. MSC702 to be 2.407 mg/ml and 21.853 mol/min/mg, respectively.

3.12 Production of HFCS

The HFCS is produced from non-sweet sources like starch or cellulose. These initial polysaccharides are cheap and are available in abundance as agricultural residues [36]. The production of HFCS is carried out in two steps which involve hydrolysis of starch into glucose followed by isomerization into fructose [37].

In our investigation, we observed 90.9% hydrolysis of starch (Fig. 13a). This saccharification rate is appreciable as it is achieved using crude enzyme. Kaneko et al. [29] have reported around 90% yield of monosaccharides by breakdown of starch using purified amylases and optimizing the pH. An isomerization of 40.8% was achieved using crude GI from isolate KP (Fig. 13b). Researchers have reported conversions around 40% [29] using purified enzyme. The isomerization step depends on the breakdown of starch polysaccharide into glucose units. The production of excessive dextrans, maltoses or other residual sugars during saccharification can decrease the efficiency of isomerization. Both the steps of HFCS production were carried out at similar pH which is advantageous as it cut down tedious procedures of pH alterations. We can still achieve better results using purified enzyme for the isomerizing reaction.

Fig. 10 **a** Effect of temperature on GI activity. **b** Effect of temperature on amylase activity

Fig. 11 **a** Effect of pH on GI activity. **b** Effect of pH on amylase activity

Fig. 12 **a** Lineweaver-Burk plot for GI under varying substrate concentration. **b** Lineweaver-Burk plot for amylase under varying substrate concentration

Fig. 13 **a** Hydrolysis of starch into glucose. **b** Isomerization of glucose into fructose

3.13 End product analysis using HPLC

The HPLC analysis of HFCS revealed that it is composed of glucose and fructose. HPLC was found to be effective in detection and separation of sugars. The chromatogram revealed that the HFCS is comprised of a mixture of glucose and fructose (Fig. 14). The sharpness in peaks in our result indicates that a single molecule is eluted at a given retention time, and the HFCS is free from other impurities. The retention time of glucose was observed to be ~ 15 min and that of fructose to be ~ 18 min at the flow rate of 0.3 ml/min of solvent. The HPLC analysis confirmed that the end products formed during the HFCS production were glucose and fructose.

HFCS is a sweetener of today with wide applications in food, beverage, baking and pharmaceutical industries. Its advantages makes it a perfect alternative of sucrose or table sugar. In India, currently the HFCS has been imported majorly from different countries. Through this study, we intend to develop an indigenous technology for the production of HFCS using *Streptomyces* sp. HFCS gets absorbed in the body without the intervention of insulin which makes it suitable for use in diabetes [1]. Moreover, HFCS is a natural sugar derived from starch rich material which is converted enzymatically into HFCS without the addition of any chemicals or preservatives. HFCS has no side effects which is its another major advantage. It can be efficiently employed in bakery, canning and confectionary products and doesn't pose the problem of crystallization. Its sweetness is comparable with that of sucrose, as it also possess glucose and fructose in independent forms [35]. The table sugar or sucrose consumption on a daily basis risks the mental health causing impairment of incognitive

functions and affecting decision making ability [3]. Apart from that, sucrose is also responsible for obesity and heart-related problems. The current day sugarfree that are used in bakeries and confectionaries usually comprise aspartame that is not suitable for kids and phenylketonurics. Aspartame metabolites cause changes in hippocampus antioxidant defense system of brain causing damage to brain tissues [38]. The other sugarfree like stevia possesses a bitter taste. Fructo-oligosaccharides are also utilized as sugarfree, but their major drawback is that they are not naturally sweet and need to be combined with a sweet base like sucralose to be able to use as a sweetener.

The current scenario clearly indicates that there is a void in the market with respect to sugars which needs to be filled. The strong roots that sucrose have in our daily life need to be replaced with better alternative with lesser or no side effects. In this situation, HFCS is the key sweetener that can perfectly replace table sugar without compromising sweetness and with better and improved properties.

4 Conclusion

The advantages and widespread uses of HFCS in beverages, canning, bakery and confectionary industry make it most demanding sweetener of modern world. The implementation of enzyme is the most crucial step for HFCS production, and enzymes from different microbial sources have been tested for the better HFCS production. The present study reports the production of GI from *Streptomyces enissocaesilis* for the first time. It exhibits significant isomerization rate of 40% in crude form and without optimization which is comparable to purified enzyme reported

Fig. 14 HPLC chromatogram of HFCS produced using amylase and GI

by other researchers. A drastic increase in saccharification and isomerization after optimization is expected which will prove the technique to be highly economical. Here, we also emphasize on the significant rate of isomerization at 70 °C as compared to commercially available GI which has lower temperature optima. The industrial processes require enzymes working at high temperatures; therefore, applicability of newly reported GI is high. Our study also accentuates on extracellular production of GI as compared to other reports on intracellular production which further is an advantage for increasing the economic viability.

Both the steps involved in HFCS production were efficiently carried out at the same pH. This advantage can be exploited at industrial scale as it reduces the cost and efforts of manipulating pH at different stages. This study might open new doors for the establishment of HFCS industries in India.

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Authors contributions TAS conducted the research and wrote the paper. AJ analyzed the data, and SB designed the experiments. TAS, AJ and SB drafted the final manuscript. All authors have read and approved the manuscript.

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