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Construction of new stable strain over-expressing the glucose isomerase of the *Streptomyces* sp. SK strain

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Abstract

In order to over express the *xylA* gene of *Streptomyces* sp. SK strain, it was cloned under the control of the constitutive *ermE-up* promoter. This construct was integrated through site-specific recombination process into the chromosome of a *Streptomyces violaceoniger* glucose isomerase deficient strain using the non-replicative vector pTS55. The resulting CBS4 strain shows a perfect stability in the absence of selection pressure. Its glucose isomerase activity was about four and nine-fold greater, than that obtained from *Streptomyces* sp. SK, respectively fully induced or not by xylose.

Keywords: Glucose isomerase; Over-expression; CBS4 strain; *ermE-up* promoter

1. Introduction

Xylose isomerase (D-xylose ketol isomerase EC 5.3.1.5) catalyses the reversible isomerization of D-xylose into D-xylulose. It is also referred to as glucose isomerase (GI) because of its ability to convert D-glucose to D-fructose. This property is widely exploited industrially for the production of high-fructose syrup from starch [1].

Isomerization at high temperature increases the reaction rate and allows the shift of equilibrium between glucose and fructose toward the later [2]. This is useful in developing a one step process for the conversion of starch to HFCS and avoided the multiple enzymatic steps at varying temperatures and reaction conditions [3]. Hence, many thermoactive GIs were isolated and studied including those from *Thermotoga maritima* [4], *Thermus caldophilus* [5] and *Thermus thermophilus* [6]. Certainly, these interesting features must be coupled to the enzyme's tolerance to acidic pH because of the formation of bitter (browning) sub-products (mannose, psicose, and other acidic compounds) under the condition of higher temperature and alkaline pH [7]. Moreover, glucose syrup production process from starch involves enzymes acting at acidic pH range (α-amylase, Glucoamylase). Hence the pH of the resulting starch hydrolysis should be adjusted before making use of glucose isomerase enzyme.

For a better industrial application, the properties of some described enzymes were ameliorated by genetic engineering. For example, it was demonstrated that the change of Asp56 to Asn and Glu221 to Ala, in the GI of *Streptomyces rubiginosus*, decreased the optimum pH of this enzyme and enhanced its catalytic efficiency [8].

In spite of the existence of several works describing the improvement of the GI performances, no recent studies have been reported concerning the enhancement of the strains productivity. Such approach is certainly of importance due to the potential use of improved strains in biotechnological process, especially those producing glucose isomerases.

We have previously reported the cloning and the preliminary characterization of a thermostable glucose isomerase (SKGI), isolated from a new thermophilic *Streptomyces* sp. SK strain, which has an optimum temperature of about 90 °C and a wide pH range with an optimum of 6 and 6.5 at 60 and 90 °C, respectively [9]. These properties coupled to some aa sequence originalities make it attractive for an industrial application [10]. Furthermore, the enzyme purification as

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well as the implication of alanine 103 residue in biochemical properties of the purified enzyme was demonstrated via site directed mutagenesis and molecular modelling techniques [11].

In this work, we describe the construction of a *Streptomyces violaceoniger* derivative stable strain, constitutively over-expressing the *Streptomyces* SK glucose isomerase activity.

2. Materials and methods

2.1. Bacterial strains and media

The *Streptomyces* strains used were *Streptomyces violaceoniger* SV10 (*xylA*, *xylB* mutant) [12], *Streptomyces* SK [9] and CBS4 (this work). Culture of *Streptomyces* SK strain was carried out at 45 °C. SV10 and CBS strains were grown at 30 °C. Cultures were done in gruel 30 g/l, yeast extract 5 g/l and agar 20 g/l, pH 6.8 [9] or in TSYE liquid media: 30 g/l Tryptic Soy Broth and 5 g/l yeast extract. Thisostrepton (Sigma) was added when appropriate at 50 μg/ml.

E. coli HB101 (*F⁻ hsdS2 recA13 ara⁻ 14 pro A2 lacY1 galk2 rps L20 xyl⁻5 mtl sup E44*) and DH5α (*supE*44 Δ*lac*U169 (Φ80 *lac*ZΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96, *thi*-1 *relA*), used as host strains, were grown in Luria–Bertani (LB) medium supplemented with ampicillin 100 μg/ml.

2.2. PCR and plasmid constructions

pIJ4070 is a plasmid containing the *ermE*-up constitutive promoter from *Streptomyces erythraeus* [13], pTS55 (generously obtained from Dr. Michel Guerineau, Université Paris Sud France) is a *Streptomyces* integrative vector [14] carrying the integrase and *attP* elements of pSAM2 and pBSK3 (as a source of the *xylA* SK gene) were used in this work.

The plasmids pMM6 and pMM9 (this work) were constructed as follows: a 1300 bp fragment containing the *xylA* with its native promoter was obtained by PCR using oligonucleotides O1 (from position −37 to −10: 5′-CTGACGAAATAGTCGCAAGCGAGCAAGG-3′), O2 (5′-GGAGATCGACCGCGCGTACCCCCTCGTC-3′) and pBSK3 plasmid as template. The cycling parameters were 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 60 s, and 72 °C for 120 s. PCR product was purified after electrophoresis in 1.4% low melting temperature agarose gel (NuSieve® GTG® Agarose). Subsequently, this fragment was cloned into the pIJ4070 vector and introduced into *E. coli* DH5α strain, resulting in plasmid pMM6. In this construction, the *xylA* SK was placed under the control of the *ermE*-up constitutive promoter from *Streptomyces erythraeus* [13]. Then, the *Bgl*II fragment carrying the insert *ermE*-up *xylA* SK from pMM6 was cloned into the *Streptomyces* integrative vector pTS55 linearized by *Bam*HI leading to the pMM9. This plasmid, firstly obtained within *E. coli* HB101 strain, was used for the construction of the CBS4 strain.

2.3. Transformation and recombinant DNA techniques

For *E. coli* manipulation, standard procedures were used [15]. For *Streptomyces* strains, molecular biology experiments and transformation approaches were performed according to Hopwood et al. [16] and Marcel et al. [12].

2.4. Preparation of free cell lysate and protein quantification

The *Streptomyces* CBS4 and SK strains were grown in TSYE during 48 h, cells were harvested by centrifugation at 8000 rpm for 10 min and the pellets were suspended in TE-buffer with 10 mM $MgCl_2$ and 1 mM $CoCl_2$. After incubation for an hour on ice in presence of 5 mg/ml lysozyme, 100 μg/ml PMSF and 1 μg/ml Pepstatin A, cells were disrupted by sonication at 4 °C for 6 min (pulsations of 3 s, amplify 90) using a vibra cell Sonicator (Fisher-Bioblock Scientific) and debris were removed by centrifugation at 20,000 rpm for 30 min. Protein concentration was determined by Bradford's method [17] using the Bovine Serum Albumin as standard.

2.5. Enzyme assays

The glucose isomerase activity was determined indirectly by using the fructose as substrate, followed by the measurement of the amount of glucose produced. The activity was assayed in a reaction mixture containing the enzyme (100 μl of an appropriate diluted purified or crude extract) with 10 mM $MgCl_2$, 1 mM $CoCl_2$ and 15% fructose, in a volume of 400 μl. In standard condition, assays were incubated for 30 min at 80 °C and the reaction was stopped by cooling the tubes on ice. The amount of glucose generated was determined by glucose-oxidase (GOD-PAP) enzyme system and A_{650} was measured after 40 min at room temperature. One unit of glucose isomerase activity is defined as the amount of enzyme needed to produce 1 μmole of product per min under the assay conditions.

3. Results and discussion

3.1. Construction of the CBS4 strain and study of its stability

The expression of the *xylA* gene of the *Streptomyces* SK strain was placed under the control of the *ermE-up* constitutive promoter from *Streptomyces erythraeus* [13], as described in materials and methods. This construct was inserted into the *Streptomyces* integrative vector pTS55. The resultant plasmid (pMM9) was than used to transform the SV10 protoplasts and selection of transformants was achieved with thiostrepton at 50 μg/ml (Fig. 1). The analysis of the few obtained clones showed that they are all xylose positive. One of these clones, called CBS4 was sub-cloned

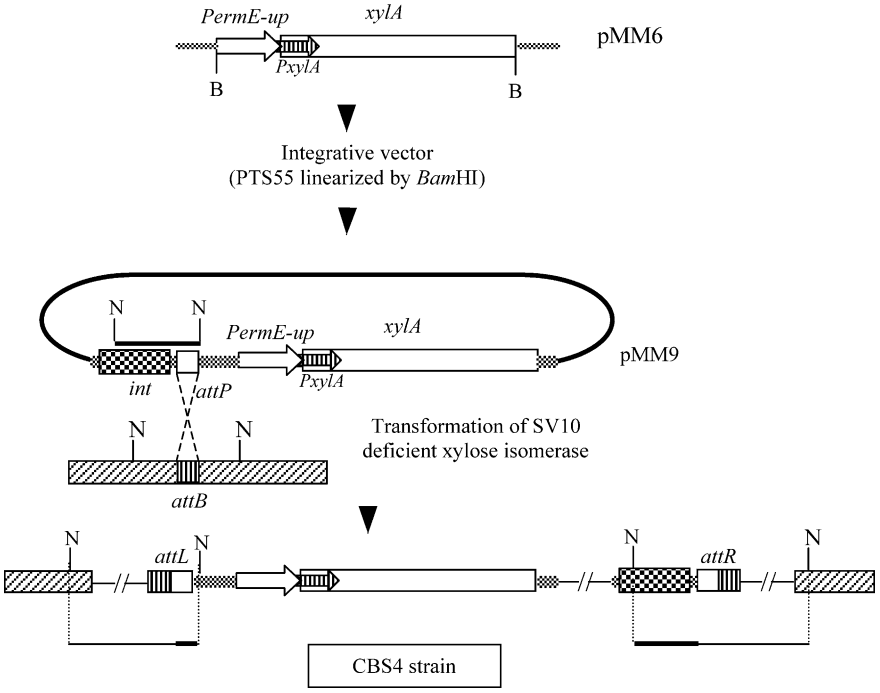

Fig. 1. Schematic representation of CBS4 strain construction. Promoters are indicated by open arrows. *PermE-up*, constitutive promoter of *Streptomyces erythraeus*, *PxylA*, native *xylA* gene promoter. Thick black line represents the 850 bp-*Nco*I fragment used as probe in Southern analysis; B, *Bgl*II; N, *Nco*I. *int*, integrase gene of pSAM2; *attP* and *attB* are respectively the attachment sites of pSAM2 plasmid and SV10 strain.

five times on a solid media in absence of thiostrepton followed by cultivation for more than 100 generations in liquid media in absence of selection pressure. The plating of this culture on solid media, on both with and without selective pressure, showed that 100% of the colonies were thiostrepton resistant and xylose positive.

Furthermore, the chromosomal structure of the CBS4 strain was checked by Southern Blot hybridization using the 850 bp *Nco*I-fragment internal to pTS55 as probe, which contain the C-terminus region of the *int* gene and the *attP* sequences of pSAM2. As expected and illustrated in Fig. 1, we detected *Nco*I hybridizing bands of 850 bp in the case of pTS55 and pMM9 plasmids and two larger bands (of nearly 2500 and 3000 bp) with the CBS4 strain (Fig. 2). The third observed weak band of about 4500 bp, could be the result of partially digested DNA.

3.2. Comparison of the glucose isomerase activity of CBS4 and Streptomyces SK strains

The mycelial intracellular glucose isomerase activity of the CBS4 and *Streptomyces* SK strains were determined from 48 h old cultures supplemented with various xylose concentrations (Table 1).

As shown in Table 1, glucose isomerase activity of the CBS4 strain is nearly constitutive in absence of xylose and slightly higher when the inducer was added. This activity is of about four-fold higher than that of the SK strain fully induced by xylose. This ratio reaches approximately nine-fold when we compare the activities of both strains without inducer (xylose).

The slightly differences between the glucose isomerase activities observed for the CBS4 strain in presence of different concentrations of xylose could be explained by the presence

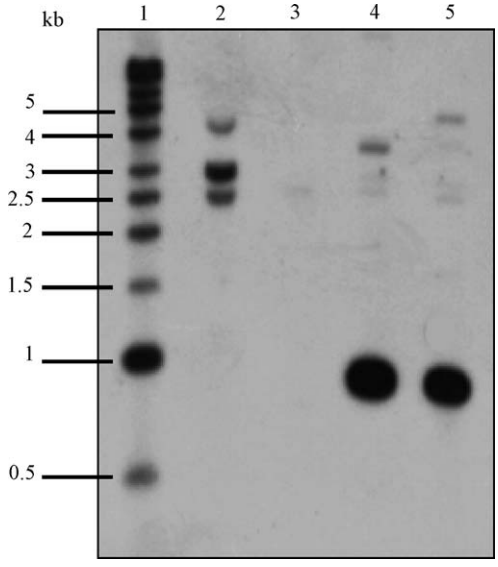

Fig. 2. Southern blot analysis of genomic DNA of SV10 and CBS4 strains. The radiolabelled 850 bp *Nco*I-*Nco*I internal fragment of pTS55 carrying the *attP* was used as probe. lane 1, radiolabelled 1-kb ladder, (lanes 2 and 3, genomic DNA digested by *Nco*I of respectively CBS4 and SV10 strains, lanes 4 and 5 are respectively pTS55 and pMM9 DNA digested by *Nco*I).

Table 1
Glucose isomerase activity of *Streptomyces* SK and CBS4 strains measured after 48 h of culture in presence of different concentrations of xylose and glucose

	Xyl: 0	Xyl: 5	Xyl: 10	Glu: 10	Xyl: 10, Glu: 10
S. SK	30	78	100	28	77
CBS4	310	342	390	350	350

This activity is arbitrarily fixed at 100% for *Streptomyces* SK fully induced by 10 g/l of xylose, corresponding to a specific activity of 0.41 U/mg. Values are the means of triplicate independent measurements.

of the native promoter of the *xylA* SK gene in addition to that conferred by the *ermE-up* promoter. In fact, comparing to the SK strain, we could notice that the *xylA* promoter is semi-inducible by xylose with a maximum of induction in presence of 5–10 g/l. The high activity of GI obtained from the CBS4 in absence of xylose is particularly interesting for the exploitation of this strain at industrial level due to the higher cost of xylose (or derivatives) comparing to that of glucose.

In conclusion, the newly constructed CBS4 strain, with improved productivity gives the opportunity to ameliorate the production pathway of high-fructose syrup from starch hydrolysis. This work takes more importance when one considers the acidotolerance and thermostability of the overproduced biocatalyst.

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