Optimization, purification and characterization of α-glucosidase inhibitors from *Streptomyces costaricanus* EBL.HB6 isolated in Vietnam

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Research Article

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Abstract

Background

Diabetes, a disease that has been a great burden of the treatment cost for patients and society. There are many drugs have been used to cure this disease available on the pharmaceutical market. One of the most prevalent source to produce these compounds are microorganism. Among them, *Streptomyces* sp. are popular microorganisms used for the production of α-glucosidase inhibitors (AGIs).

Methods and Results

In this study, different cultivation conditions were optimized to enhance the production of AGIs. Purification and evaluation of AGIs from *S. costaricanus* EBL.HB6 were also performed. Our results demonstrated that *Streptomyces costaricanus* EBL.HB6 had the highest α-glucosidase inhibitory activity among 6 *Streptomyces* sp. strains were isolated in Vietnam. The 16S rRNA sequencing of isolating HBC6-2 indicated 99% identity to the corresponding sequence of *Streptomyces costaricanus,* and was registered on GenBank with the code MT 453944.1. *Streptomyces costaricanus* EBL.HB6 was able to produce melanin yellow pigment, and its aerial and substrate mycelia have brown and yellow-grey pigment on ISP2 cultivating medium, respectively. The α-glucosidase inhibitory activity of the supernatant was increased by a factor of 1.2 under optimal conditions (media containing 1.5% glucose, 1.2% yeast extract at 28°C, initial pH of 6.5, and culture time for 120 h) in comparison with the initial media and condition. The purified efficacy of a-glucosidase inhibitors was 5% with a retention factor of 0.71 on thin-layer chromatography and IC_{50} value of 9.59 mg/mL.

Conclusions

Streptomyces costaricanus EBL.HB6 strain was selected, purified and evaluated for its highly producible of α-glucosidase inhibitors.

Introduction

The *Actinomycetes* sp. belongs to the group of endophytic microorganisms that can produce a myriad of inhibitors against pathogenic microorganisms [1]. Therefore, several studies have investigated its role as a bio-control factor including promoting plant growth, reducing the risk of the infectious pathogen, and enhancing the viability of plants under different conditions [2]. *Actinomycetes* took a major part in the population of root microorganisms so they easily transmit to the plant and become an endophytic body. The population includes both *Streptomyces* and non-*Streptomyces* appear in plant tissues [3]. A large number of publications in microbial compounds have reported that 45% of substances originated from *Actinomycetes*, 38% were from mushrooms, and 17% were from bacteria. *Actinomycetes* are one of the most important microorganisms due to the majority of their secondary metabolites including enzymes, antibiotics, antifungals go to industrial, agricultural, and pharmaceutical markets [4].

Most of these biologically active substances were discovered from terrestrial *Actinomycetes* which are recognized as a reliable resource for the production of antibiotics and new pharmaceutical compounds. The *Actinoplane* sp. and *Streptomyces* sp. are the most popular species that capable of producing α-glucosidase inhibitors (AGIs) such as acarbose of *Actinoplanes* [5, 6], AGIs from *Streptomyces* sp. [7, 8], and other aminoglycosides, anthracyclin, glycopeptide…[9].

The ratio of discovering pharmaceutical compounds from endophytic *Actinomycetes* is higher than soil-borne and plan *Actinomycetes*. A new antibiotic, Naphthomycin-K, was first discovered from endophytic *Streptomyces* sp. from *Maytenus Hookeri*-a medicinal plant that has effects against cancer. Two compounds, 5,7-dimethoxy-4-phenylcoumari and 5,7-dimethoxy-4-p-methoxylphenylcoumarin, strongly inhibited cancer growth which is frequently isolated from different plant species. Recently, they are also found in endophytic *S. aureofaciens* [10]. Methyllelaiophylin of *S. melanosporofaciens* inhibited α-glucosidase with IC_{50} at 10 µM [11]. Kaur (2016) isolated several antibiotics from endogenous *Streptomyces, Micromonospora, Microbiospora*, *Nocardia* on the leaves of the neem tree (*Azadirachta indica* A. Juss.) [12]. A new active substance was extracted from *Streptomyces* sp. OUCMDZ-3434 isolating from algae samples. These are 2 new AGIs that have been discovered. Wailupemycins H (1) with Ki/IC_{50} were 16.8/19.7 µM, and Wailupemycins I (2) with Ki/IC_{50} were 6.0/8.3 M [9]. Wei (2017) isolated 24 compounds from the fermentation broth of *S. xanthophaeus* that were numbered from 1–24, and their chemical formula was elucidated by NMR. The authors have identified 3 compounds including daidzein, genistein, and gliricidin which inhibited α-glucosidase *in vitro* with IC_{50} were 174.2; 36.1 and 47.4 µM, respectively. The results showed the inhibitory activity was higher than that of acarbose [13].

In this study, HBC6-2 was choosed for its high production of AGIs from 6 different strains that were isolated from orange trees originated from Hoa Binh, Viet Nam. We next optimized culture conditions to increase the production and purified AGIs for further study.

Materials And Methods

Microorganism

The *Streptomyces* sp. strain was provided by Soil Microbiology of Laboratory, Institute of Biotechnology, Vietnam Academy of Science and Technology including HBC3-2, HBC5-1, HBC6-2, HBR5-1, HBR9-6, and HBR10-2. This *Streptomyces* sp. strains were isolated from the samples of root, stem, and leaf of Cao Phong orange, Hoa Binh, Vietnam.

The strain *Streptomyces* sp. was cultivated in ISP2 medium (g/L): 4 yeast extract, 10 malt extract, 4 dextrose, and pH 7.2. The condition for culturing *Streptomyces* sp. was 28°C, shaking at 200 rpm and 120 h.

Chemical reagent

Acarbose, dimethyl sulfoxide (DMSO), and *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) were obtained from Sigma-Aldrich. K_2HPO_4, KH_2PO_4, Na_2HPO_4, NaH_2PO_4 were obtained from Merck. Alpha-glucosidase, glucose, dextrose, glycerol, yeast extract, and malt extract were purchased from Biobasic INC (Canada). Sephadex^{TM} G-75 was purchased from GE Healthcare Bio-Sciences AB (Sweden). All other chemicals are analytical grade, otherwise stated.

Fermentation

The *Streptomyces* sp. strain was streaked on the agar plate. After 7 days, colonies appeared with yellow-brown color. A single one was inoculated in a 100 mL propagation medium. After 72 h, it was fermented in a medium consisting of (g/L): 12 yeast extract, 15 glucose, pH 6.5 in 120 h at 28°C and 200 rpm to collect the fermentation broth.

Enzyme Assay

The tests were performed in a 96-well microplate setup with slight modifications [14]. A reaction mixture of 100 µL of 1-2 U/mL α-glucosidase was dissolved in 0.1 M sodium phosphate buffer and mixed with 10 µL fermentation broth (or $CHCl_3$/Act extract) and 40 µL of phosphate buffer solution, and then was subjected to pre-incubation in 5 min at 30°C. Continuously, 100 µL of 4-nitrophenyl-α-D-glucopyranoside 0.1 M in phosphate buffer was added as substrate after pre-incubation. Next, the samples were incubated at 30°C for 10 min to allow α-glucosidase to react with 4-NPG and produce 4-nitrophenol. After the incubation, the formation of 4-nitrophenol in each well was measured at 405 nm. The inhibitory activity was calculated using

$$\% \text{ Inhibition} = \frac{\Delta Ac - \Delta As}{\Delta Ac} \text{ x } 100$$

ΔAc : The change in measurement of OD value before and after incubation for 5 min of control.

ΔAs : The change in measurement of OD value before and after incubation for 5 min of the sample.

DNA isolation, identification of chosen strain

The 16S rDNA sequencing method was used to identify the isolated strain. Genomic DNA isolation was used to extract DNA from a potent AGIs strain [15]. The isolated DNA was amplified by PCR. The conserved gene of 16S rRNA was amplified by using 9F (5'-AGAGTTTGATCCTGGCTC-3') as the forward primer and 926R (5'-CCGTCAATTCCTTTGAGTT-3') as the reverse primer. The amplified gene was sequenced on ABI PRISM 3100 Avant Genetic Analyzer. Sequence alignments were analyzed using the program MegAlign DNAStar

Culture conditions optimization

Time and temperature culture

Unless otherwise stated, *S. costaricanus* EBL.HB6 was cultivated in a 250 mL flask with 50 mL ISP2 medium at 28°C with 200 rpm shaking and pH of 7.2. Extracellular extract from the culturing medium was obtained and α-glucosidase inhibitory activity tests were carried out after 24, 48, 72, 86, 120, 144, 168, and 192 h to select the best culture time.

To select optimum temperature for the AGIs production, *S. costaricanus* EBL.HB6 was cultured at different temperature from 28 to 37°C on ISP2 medium at pH 7.2.

Carbon source and concentration

The effect of various additional carbon sources on the a-glucosidase inhibitors production including: starch soluble, sucrose, maltose, glucose, dextrose, and lactose at the concentration of 0.4% (w/v) was investigated. *S. costaricanus* EBL.HB6 was grown in 250 mL shaking flasks containing 50 mL of the medium with 1% of malt extract, 0.4% yeast extract (w/v), and 0.4% (w/v) of different carbon sources.

The levels of carbon source giving the highest AGIs production varied from 0.4 to 3.5% (w/v).

Nitrogen source and concentration

The effect of various additional nitrogen sources including peptone A, peptone B, $(NH_4)_2SO_4$, yeast extract, and malt extract at the concentration of 1.0% (w/v) was employed. In addition, the ISP2 medium was used to as a control sample.

The levels of nitrogen giving the highest AGIs production varied from 1.0 to 1.8% (w/v).

Initial medium pH

Fermentation in different pH culture media (pH 6, 6.5, 7, 7.5, and 8) was used to determine the ideal initial pH in flask culture, which was changed with either 1 N NaOH or 1 N HCl. All the tests were conducted in triplicate.

Purification of the α-glucosidase inhibitors

The *S. costaricanus* EBL.HB6 was cultured in the optimized fermentation media at pH 6.5, incubated at 28°C for 120 h. 100 mL of the cell culture broth was centrifuged at 12500 rpm in 15 min. The fermentation broth was extracted with ethanol (80%) for 30 min, then centrifuged at 12500 rpm in 15 min. The collected liquid was lyophilized. Alpha-glucosidase inhibitory activity of the re-solubilized solution was determined, and then applied to a Sephadex^{TM} G-75 column (0.6 × 26 cm) pre-equilibrated with 0.02 M phosphate buffer (pH 6.8) at a flow rate of 25 mL/hr until the OD 280 nm was < 0.01. The column was then eluted with 0.02 M phosphate buffer (pH 6.8). The eluted fractions of 2 mL were collected (20 fractions). The active fraction was lyophilized and alkalized to pH 10-11 by 0.1 N NaOH after re-solubilized by phosphate buffer. 10 mL of solution was extracted with n-butanol (1:1; v/v) three

times. The upper phase was obtained by centrifugal at 4000 rpm in 15 min to collect n-butanol extract residue.

The n-butanol extract residue was re-solubilized in 30 ml of $CHCl_3$/Act solvent (1:1; v/v), at 40°C, overnight, and then centrifuged at 4000 rpm for 15 min to remove residue. The extract was evaporated dissolved in DMSO.

The purified AGIs were tested and detected by thin-layer chromatography (TLC) as well as infrared spectroscopy to determine the presence of the substance group. 5 µL of samples were chromatographed on TLC plates (Merck, Germany) with solvent (n-butanol: acetone: H_2O; 5:1:4; v/v/v), then they were sprayed with iodine.

Evaluation of the IC_{50} value

The IC_{50} value was obtained by examination of the inhibition activity of α-glucosidase of purified AGIs at different levels: 5-60 mg/mL. The line graph equation was established as a function of the purified AGIs concentration (x) and inhibitory activity (y) [16]. The IC_{50} value is the level of purified AGIs value that inhibit 50% α-glucosidase activity.

Statistical analysis

All measurements were carried out in triplicate. The means were presented for the averages of experiments.

Results

Screening of α-glucosidase inhibitory activity for *Streptomyces* sp. strains

The fermentation broth of 6 strains of *Streptomyces* sp. was examined with α-glucosidase activity. *Streptomyces* sp. HBC6-2 displayed the highest α-glucosidase inhibitory activity by 68.98% among *Streptomyces* sp. strains (Fig. 1a). The strain *Streptomyces* sp. HBC6-2 produced melanin yellow pigments, non-fragmenting substrate mycelia (hypha diameter around 10 mm), and formed spiral spores containing 10-50 spores. The color of the aerial and substrate mycelia of *Streptomyces* sp. HBC6-2 was gray-brown and yellow-brown (Fig. 1b).

16S rRNA sequencing of *Streptomyces* sp. HBC6-2 was identified. BLAST results of 16S rRNA from HBC6-2 strain indicated that this 16S rRNA segment showed 99.69% identity with 16S rRNA from *S. costaricanus* MR7 (KY753206), 99.59% to *S. costaricanus* MJM5482 (FJ799179) (Fig. 1c). It was registered on GenBank with the code MT 453944.1.

Culture conditions optimization

To determine the optimal conditions for producing AGIs from *S. costaricanus* EBL.HB6, the culture parameters such as culture time, temperature, carbon, and nitrogen of source at different levels and initial pH were investigated.

Effect of culture time and temperature

The incubation time for AGIs production by *S. costaricanus* EBL.HB6 was carried out in the ISP2 medium at 28°C with shaking 200 rpm, and an initial pH of 7.2. After 120 h of culture, the a-glucosidase inhibitory activity of extracellular extracts of *S. costaricanus* EBL.HB6 reached maximal values of 70.15%. Then, it slightly decreased and after 192 h of culture as low as 42.63% (Fig. 2a).

The temperature did not significantly affect the AGIs activity of the *S. costaricanus* EBL.HB6. This strain had stable activity in the temperature range 28-37°C, however, it is capable of producing highly active α-glucosidase inhibitors at 28°C (Fig. 2b).

Effect of carbon source at different levels

Glucose-containing medium showed the highest α-glucosidase inhibitory activity of 72.42%, followed by other medium supplemented with sucrose (44.68%); malt extract (32.05%). However, starch soluble and maltose-containing medium showed the lowest inhibition activity (Fig. 2c).

Dextrose was replaced by glucose dramatically increased the AGIs production by *S. costaricanus* EBL.HB6 from 68.3% in the media containing 0.4% (w/ v) of glucose to the maximum 76.25% at glucose (1.5 %), and then gradually decreased to 57.5% in the medium containing 3.5% of glucose (Fig. 2d).

Effect of nitrogen source at its concentration

Culture medium supplementing different with nitrogen source exhibited different α-glucosidase inhibitory activity (Fig. 3a). Yeast extract adding media enhance the inhibition activity of the crude extract by 77.07%. In contrast, Peptone B and malt extract supplementing media exhibited a lower inhibition activity of 51.44% and 52.72%, respectively, and the lowest one is $(NH_4)_2SO_4$ containing media.

Especially, the fermented media only containing of yeast extract (1.2%) increased α-glucosidase inhibitory to 87.1% while ISP2 media was reached 68.98% (Fig. 3b).

Effect of initial pH

Five different initial pH 6, 6.5, 7, 7.5, and 8 were examined for their effect on AGIs production by *S. costaricanus* EBL.HB6. The optimal initial culture pH for AGIs production by *S. costaricanus* EBL.HB6 was 6.5 (Fig 3c). At this pH, the α-glucosidase inhibitory activity of extracellular extracts of *S. costaricanus* EBL.HB6 reached maximal inhibitory activity of 83.58%. An initial culture pH lower or higher than 6.5 reduced AGIs production by *S. costaricanus* EBL.HB6. These findings

suggested that the pH medium has a considerable impact on AGIs production by *S. costaricanus* EBL.HB6.

Determination of α-glucosidase inhibitory activity under optimal conditions

The *S. costaricanus* EBL.HB6 strain was grown in the optimal medium containing 1.5% of glucose, 1.2% of yeast extract, at 28°C with initial pH of 6.5 for 120 h. The result showed the α-glucosidase inhibitory activity of extracellular extracts of *S. costaricanus* EBL.HB6 was 84.97% (Fig. 3d). These results were 1.2 times higher than those obtained before optimization (69.77%).

Purification of a-glucosidase inhibitors

The ethanol extract of *S. costaricanus* EBL.HB6 was purified by passage over a Sephadex^{TM} G-75 column. These fractions were tested for α-glucosidase inhibitory activity and all were positive (Fig. 4a). Fraction 9-13 showed the highest α-glucosidase inhibitory activity of 84% (fraction 10) and 50% to 70.53%, respectively. Fractions 10 were then extracted by n-butanol three times and n-butanol extract residue was re-solubilized in $CHCl_3$/Act solvent to obtain purified inhibitors.

The purity of the purified inhibitors was assessed by TLC methods. A spot with Rf=0.71 was observed (Fig. 4b- land 4) and the yield of the purification process was 5% and exhibited inhibition of α-glucosidase activity was 45.47% (Table 1).

We next investigated the α-glucosidase inhibitor activity of purified AGIs by measuring the IC_{50} value. The purified AGIs were dissolved in DMSO at different levels. The IC_{50} of purified AGIs was 9.59 mg/mL (Fig. 4c).

The FT-IR spectrum showed that major functional groups appeared such as Ar-H vibration in aromatic ring, imide region, C=C oscillation in the aromatic ring, and oscillation outside mp Ar-H (Fig. 4d).

Discussion

Several studies reported that actinobacteria were more prevalent in the discovery of α-glucosidase inhibitors. Abdulkhair (2018) isolated 55 strains of marine *Actinomycetes*, of which, only 7 strains were found to have α-glucosidase inhibitory activity [17]. S Ganesan, S Raja, P Sampathkumar, K Sivakumar and T Thangaradjou [18] identified 41 bacteria strains that exhibited glucosidase inhibitory activity from 181 isolated strains of marine actinobacteria [18]. The study by Cansigno and his colleagues discovered that *Veracruz* seaweed was capable of synthesizing α-amylase and AGIs [19].

The culture duration of *S. costaricanus* EBL.HB6 produced the AGIs shorter than *Streptomyces* sp. strain OUCMDZ-3434 (8 days) [9], possibly because of *S. costaricanus* EBL.HB6 produced the secondary metabolite during the growth and development of *Actinomycetes*. In which, *Streptomyces* sp. OUCMDZ-3434 produced a phenolic compound that takes a long time to produce. During nutrient depletion, metabolites are generally generated during the late growth stage of bacteria [20]. This finding was in

agreement with the earlier study in which in the presence of glucose, the synthesis of acarbose, AGIs used to treat diabetes type 2, was produced [6, 21].

The AGIs was purified from the culture supernatant showed α-glucosidase inhibitory activity of 45.47% and a spot with a coefficient of Rf = 0.71 on TLC (Fig. 4b, lane 4). The purified inhibitor showed the final yield of the purification procedure was 5% (Table 1).

These results of the FT-IR spectrum suggested that AGIs production *S. costaricanus* EBL.HB6 could be an alkaloid but not a protein from *Streptomyces* sp. AD7 [17], or phenolic compounds from *Streptomyces* sp. OUCMDZ-3434 [9], *S. xanthophaeus* [13], or dibutyl phthalate from the strain *S. melanosporofaciens* [22]. This result is further evidence of the diversity in AGIs production by different *Streptomyces* sp. strains. Purified AGIs from different *Streptomyces* sp. strains may be obtained the different AGIs.

In our recent study, we determined the IC_{50} of the n-butanol extract was 13.89 µg/mL from *Oceanimonas smirnovii* EBL6, while acarbose (Sigma) was 31.16 µg/mL [23]. In this study, IC_{50} value of purified AGIs was 9.59 µg/mL. This may be suggested that *S. costaricanus* EBL.HB6 had potential for the treatment of type II diabetes for further study. Previously, ethyl acetate extracted containing AGIs from *Streptomyces* sp. IPBCC. b. 15. 1539 with IC_{50} value was 0.047 µg/mL [16]; 21.17 µg/mL from *Streptomyces* sp. S2A [7]; 3.1 mg/mL *A. oryzae* N159-1 [24]; 5.625 µg/mL from *A. awamori* [25]; 1.25 µg/mL from *Streptomyces* strain PW638 [8]; 0.062 mg/mL from *Streptomyces* sp. TVS1 [26]; 500 µg/mL from *Arthrobacter enclensis* [27]. Daidzein, genistein, and gliricidin from *S. xanthophaeus* with IC_{50} values of 174.2, 36.1 and 47.4 µM, respectively [13]; two new AGIs from *Streptomyces* sp. OUCMDZ-3434 with IC_{50} values of 19.7 and 8.3 µM, respectively [9]. So, IC_{50} value may be related to the purity level of the extracts or compound.

In conclusion, the isolating HBC6-2 was chosen for its high production of AGIs from 6 different *Streptomyces* sp. strains isolated from the samples of root, stem, and leaf of Cao Phong orange and its was registered on GenBank with the code MT 453944.1.

The optimal culture medium for fermentation of AGIs from *S. costaricanus* EBL.HB6 composed of (g/L): glucose 15, yeast extract 12; pH 6.5. The optimal incubated conditions for the production were 120 h, 28°C, and shaking speed of 200 rpm. Combination of all the optimal conditions, the α-glucosidase inhibition activity exhibited the highest point at 84.97%, increased by a factor of 1.2 times in comparison with the initial condition. In addition, we successfully purified and evaluated of IC_{50} value of AGIs from *S. costaricanus* EBL.HB6. The significant inhibitory α-glucosidase activity of *S. costaricanus* suggests its potential utility as an alternative to make biosimilars products for the treatment of type II diabetes.

Declarations

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Conflict of interest

The authors declare no competing interests.

Availability of data and material

The sequence of bacteria strain was deposited in GenBank with accession no. MT 453944.1. All data generated or analysed during this study are included in this published article.

Ethics declarations

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

All authors have approved the submitted version and give consent for publication.

Author's Contribution

DTT and NTT designed the experimental setup, assisted with data analysis and manuscript preparation. MVH performed experiments of screening of *S. costaricanus* EBL.HB6. NPDN and DTMA performed optimization of α-glucosidase inhibitory activity. NTT and PTHT purified and determined on the IC_{50} value of the AGIs. DTT initiated the project, read and approved the final manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Purification efficacy of a-glucosidase inhibitiors

Purification steps	Amounts (g)	Yield (%)	a-glucosidase inhibition (%)
The fermentation broth	1.814		84.97
Sephadex^{TM} G-75 (fraction 10)	0.4261	23.49	84.0
n-butanol extract	0.1492	8.2	54.37
The purified AGIs	0.0907	5.0	45.47

Figures

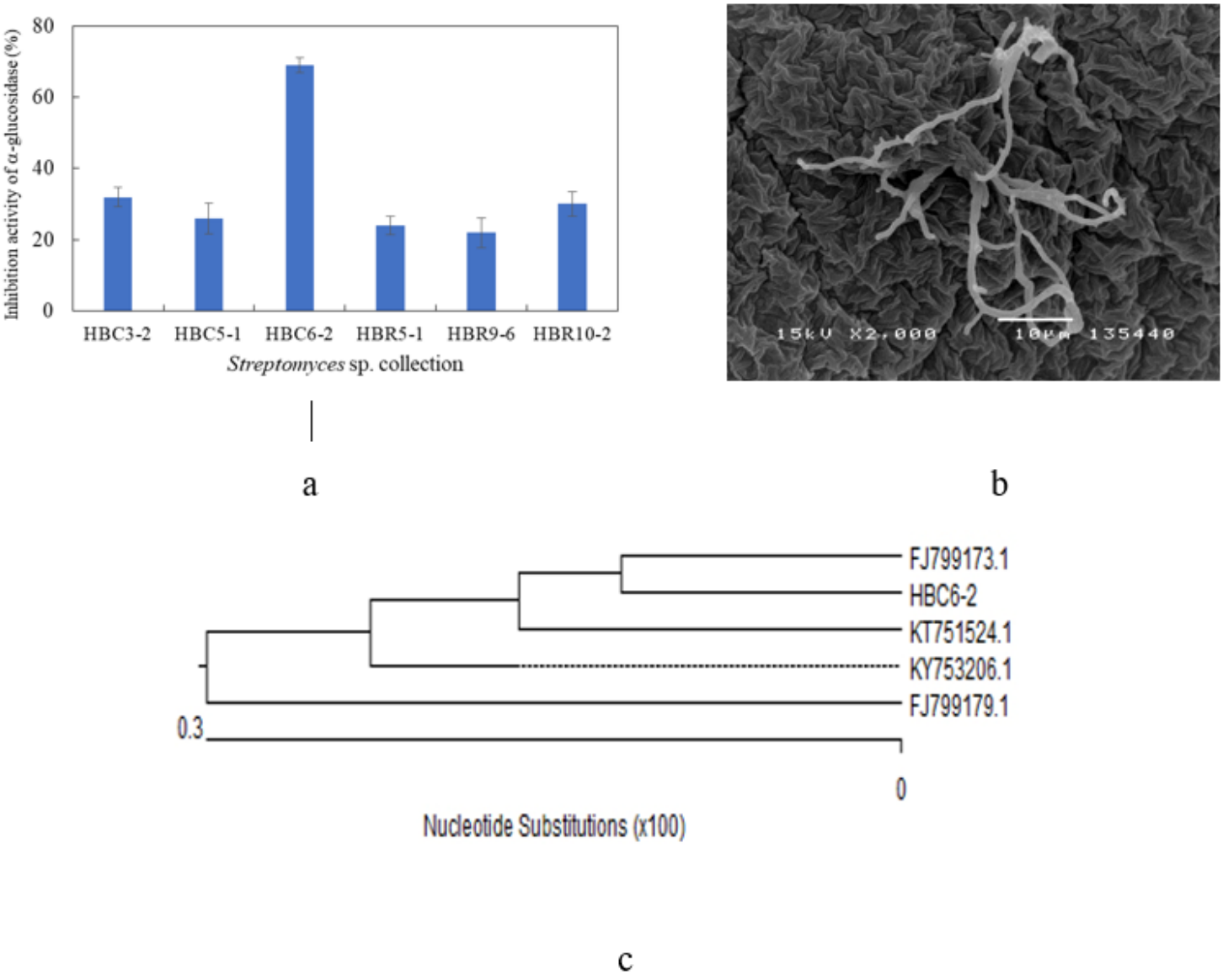

Figure 1

Screening of α-glucosidase inhibitory activity from the fermentation broth of 6 Streptomyces sp. strains (a); Substrate mycelia (magnification α 2000) (b); Taxonomy of Streptomyces sp. HBC6-2 (c)

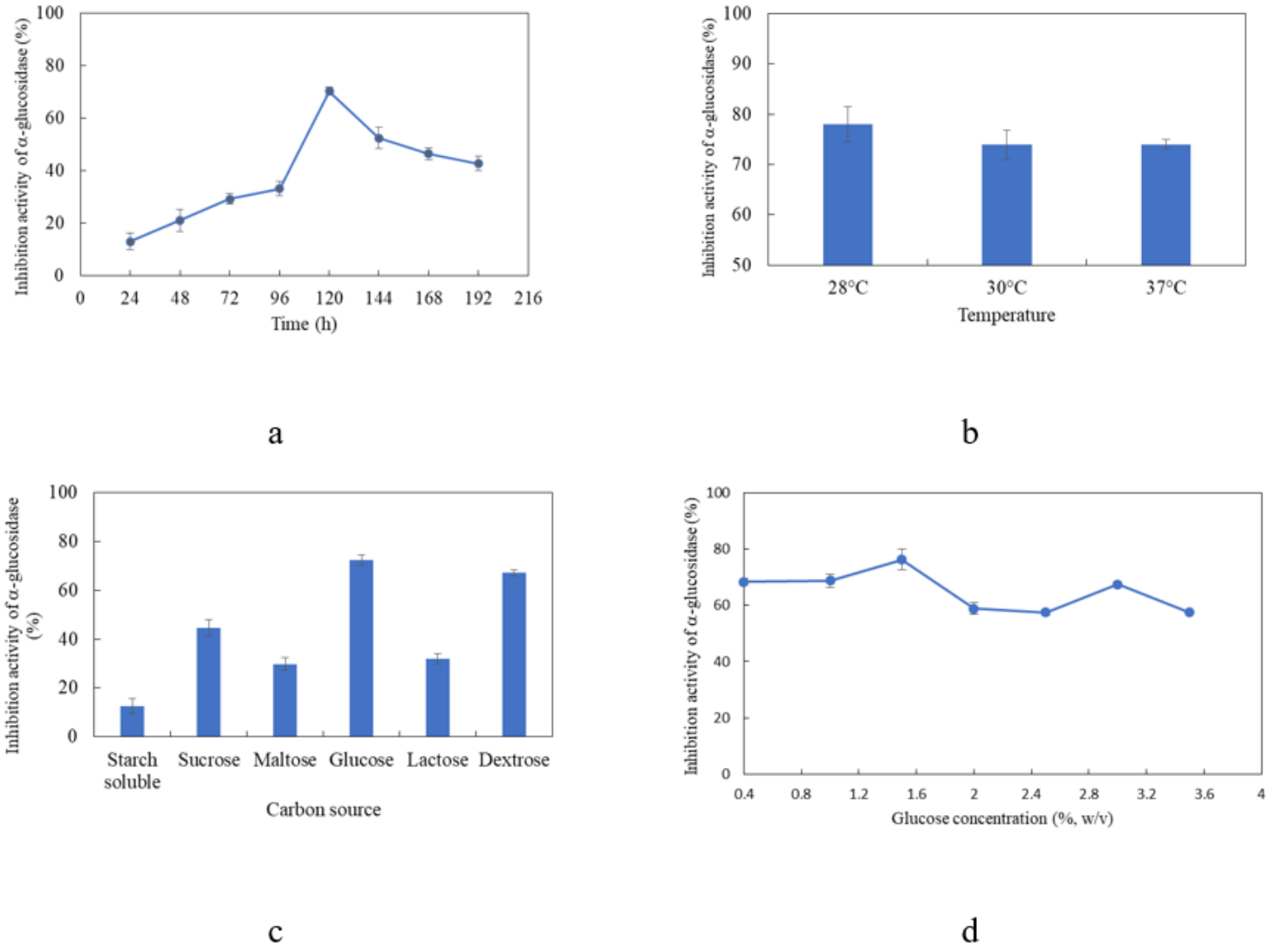

Figure 2

Effect of incubation time (a), temperature (b), carbon source (c) and glucose concentration (d) on the production of AGIs from S. costaricanus EBL.HB6

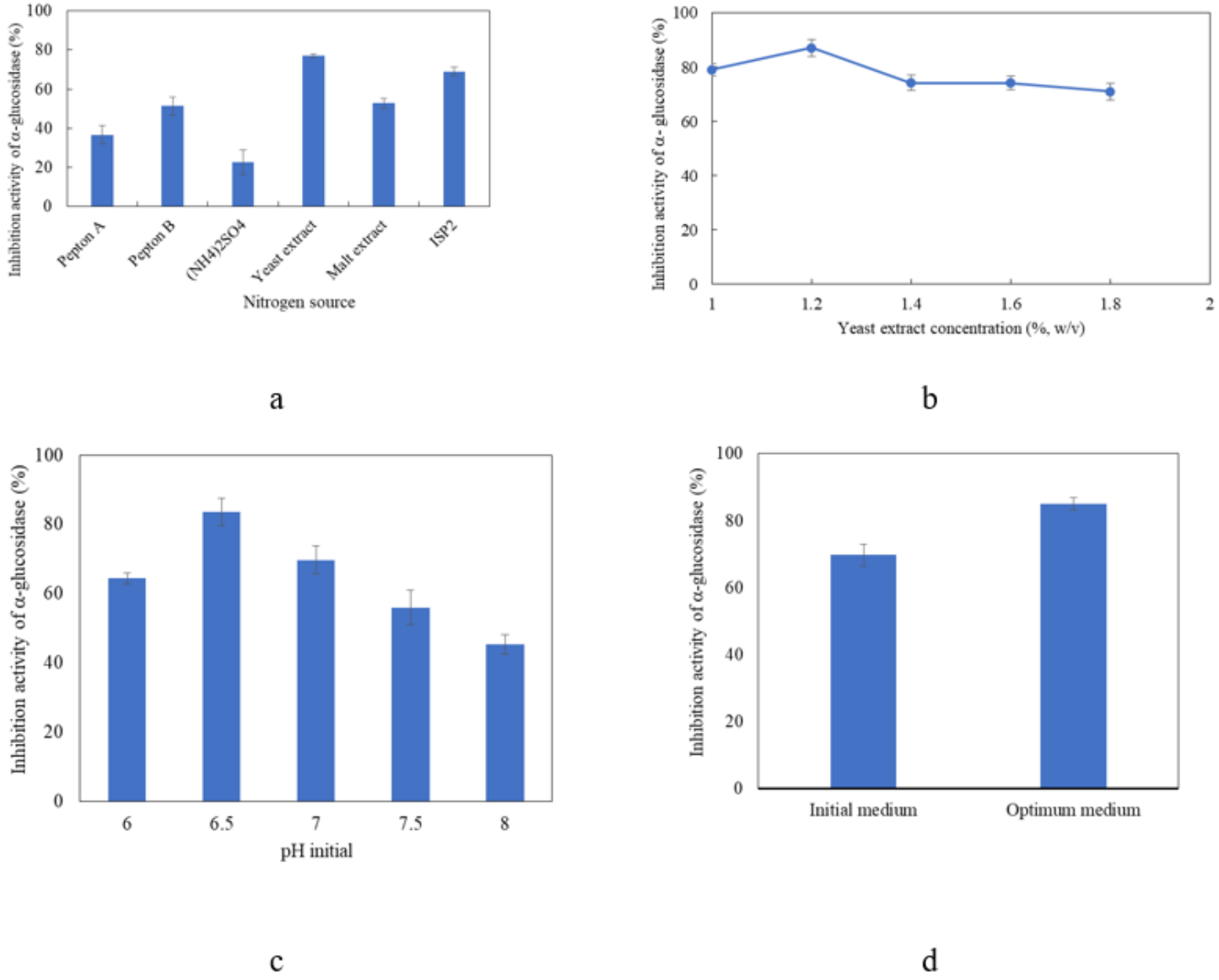

Figure 3

Effect of nitrogen source (a), yeast extract concentration (b), pH medium (c) on the production of AGIs from S. costaricanus EBL.HB6 and inhibitory activity of α-glucosidase in ISP2 medium and optimal medium (d)

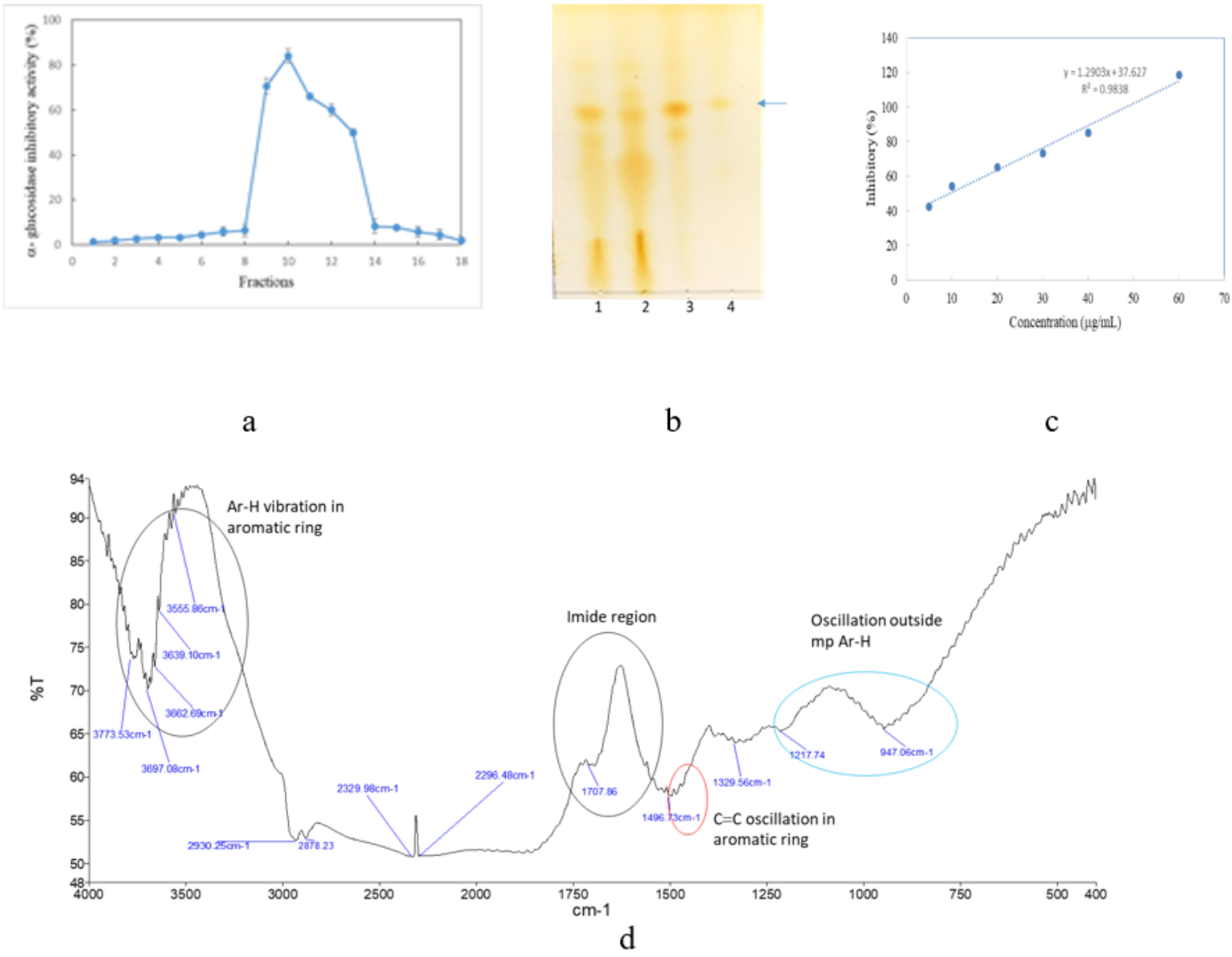

Figure 4

Fractional inhibitory activity of the samples was purified by SephadexTM G-75 column (a); The appearance of purified compound on TLC chromatography (b) (land 1: fraction 10; land 2: The fermentation broth; land 3: n-butanol extract; land 4: the purified AGIs after extracted by CHCl3/Act extract); IC50 value (c) and FT-IR spectrum of purified AGIs from S. costaricanus EBL.HB6

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Graphicalabstract.pdf