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2-Keto-gluconate production and purification by thermotolerant acetic acid bacterium *Nguyenibacter vanlangensis* KKS-R1

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ABSTRACT

2-Keto-gluconic acid (2-KGA) is a pivotal intermediate in the production process of ascorbic acid, commonly known as vitamin C. The bacterium *Nguyenibacter vanlangensis* KKS-R1 is an acetic acid bacterium (AAB) that has been selectively screened for its ability to produce 2-KGA among twenty-five bacterial isolates at high temperatures up to 40°C. This thermotolerant property makes it advantageous for various industries. Therefore, it has garnered interest for its potential application in the production of 2-KGA and purification of the 2-KGA product. This study evaluated the production of 2-KGA by N. vanlangensis KKS-R1 using 1% gluconate as the substrate. The 2-KGA was determined by thin-layer chromatography, Lanning and Cohen's method, and high-performance liquid chromatography. After fermentation in a minimal liquid medium for 24 hours, the bacterium showcased its prowess by producing 2-KGA concentrations of 7.5 g/L. However, the purity of the 2-KGA production is paramount for industrial applications. In addressing this, a rigorous chromatographic purification regimen was employed. This involved two sequential stages of ion exchange chromatography, DEAE-Sephacel and DOWEX 1X4, followed by Superdex™ S-200 column chromatography. The collective efficacy of these methodologies yielded a product with an impressive purity index of 71.02%. The 2-KGA product was then subjected to a decolorization process using activated carbon and freeze-dried. This results in the compound being a pure white powder. This investigation indicates that *N. vanlangensis* KKS-R1 shows good potential as a 2-KGA producer at high temperatures. Under proper purification techniques. These findings contribute to developing purified processes and producing 2-KGA for industrial efficiency.

Keywords: Acetic acid bacteria (AAB), Thermotolerant, 2-Ketogluconate (2-KGA), Column chromatography

INTRODUCTION

Acetic acid bacteria (AAB) are gram-negative bacteria characterized by their ellipsoidal or rod-shaped cells. They are aerobic bacteria, requiring oxygen for growth [1]. Depending on the genus, their flagella may be peritrichous or polar. Acetic acid bacteria belong to the family Acetobacteraceae within the class Alphaproteobacteria. Originally, this classification comprised two genera: *Acetobacter* and *Gluconobacter*. However, with advancements in genetic molecular techniques, which have significantly improved the classification process, 19 distinct genera of acetic acid bacteria have been identified: *Acetobacter*, *Acidomonas, Ameyamaea, Asaia, Bombella, Commensalibacter, Endobacter, Gluconacetobacter, Gluconobacter, Granulibacter, Komagataeibacter, Kozakia, Neoasaia, Neokomagataea, Nguyenibacter, Saccharibacter, Swaminathania, Swingsia,* and *Tanticharoenia* [2]. Mesophilic strains thrive optimally at temperatures between 25-30°C, while thermotolerant strains can grow in the broader temperature range, with their optimal metabolic activity occurring at 37-40°C. The optimal pH for growth is between 5.0-6.5, but these bacteria can also grow at slightly lower pH values [3, 4]. Acetic acid bacteria possess a mechanism to oxidize starting substances like alcohols, sugars, sugar alcohols, and sugar acids in an "oxidative fermentation" process. En enzymes on the cell membrane's outer surface facing the periplasm oxidize these substrates. The oxidation products are expelled outside the cell and accumulate in the culture medium [5, 6]. These properties make AAB suitable for producing various products since it allows for easy separation of the product from the bacterial cells, rapid fermentation processes, and high yields. 2-Ketogluconate (2-KGA) is an essential organic acid used in producing erythorbic acid, also known as D-isoascorbic acid. It's a stereoisomer of ascorbic acid (vitamin C) and is widely used as an antioxidant in food processing, adhering to Good Manufacturing Practice (GMP) standards. It plays a crucial role in preventing food oxidation, retaining the color, taste, and aroma of foods, and inhibiting the

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formation of carcinogenic ammonium nitrite during food processing [7-9]. In addition, it has been certified as a Generally Recognized As Safe (GRAS) compound by the U.S. Food and Drug Administration (FDA). The synthesis of 2-KGA is achieved using microorganisms through the oxidative fermentation process of D-glucose sugar. This occurs in the periplasmic space, anchoring related enzymes to the cell membrane's outer surface. The D-glucose is oxidized into D-glucono-δ-lactone, which can then be converted into D-gluconic acid. This transformation might be due to the instability of the molecule or possibly be catalyzed by the enzyme gluconolactonase [10]. Subsequently, D-gluconic acid is further oxidized by enzymes on the cell membrane, specifically by the enzyme D-gluconate dehydrogenase [11, 12], which has a cofactor known as flavin adenine dinucleotide, referred to as FAD-GADH, and the enzyme glycerol dehydrogenase [13], which has a cofactor known as PQQ and is referred to as PQQ-GLDH. FAD-GADH catalyzes the reaction to produce 2-KGA, while PQQ-GLDH catalyzes the reaction to produce 5-keto-D-gluconic acid (5KGA). Both reactions occur competitively. Either 2-KGA or 5-KGA is excreted outside the cell until the glucose is completely oxidized. However, in some strains, it has been observed that 2-KGA is further oxidized to 2,5-diketo-D-gluconic acid (2,5 DKGA) by the enzyme 2-KGA dehydrogenase. This enzyme is membrane-bound and has FAD as its cofactor [14]. Almost all industrial-scale production of 2-KGA is achieved through microbial fermentation, as chemical and enzymatic methods yield low volumetric productivity, and the catalysts or enzymes are expensive. Moreover, the products from the oxidative fermentation process of acetic acid bacteria can be utilized without cell lysis, which involves separating the cells and subsequently purifying the desired compounds using various methods based on their chemical and physical properties. Consequently, researchers have been selecting strains of acetic acid bacteria capable of producing 2-KGA at elevated temperatures and studying the purification process of 2-KGA at the laboratory scale, aiming for further development at an industrial scale.

MATERIALS AND METHODS

Microorganisms

The 25 tested strains are bacteria obtained by isolating the roots of rice plants. It can fix nitrogen and effectively dissolve phosphorus and zinc, identified using 16S rRNA gene sequence analysis [15] (Table 1).

Table 1 Bacterial isolates were identified using 16S rRNA gene sequence analysis [15] for screening of 2-KGA production.

Code Isolate	Isolate gene similarity in NCBI database	% of identify	Code Isolate	Isolate gene similarity in NCBI database	% of identify
TBRC4639	*N. vanlangensis* TBRC4639 ^{T}	100.00	TBRC 479	*G. azotrocaptans* TBRC 479 ^{T}	100.00
KKS-R4	*N. vanlangensis* TN01LGI	100.00	AR-R1	*G. diazotrophicus* LMG 7603	99.96
KKS-RZ1	*N. vanlangensis* TN01LGI	96.67	PS-RZ1	*A. methanolica* NBRC 104435	99.92
NKS-RZ1	*N. vanlangensis* TN01LGI	99.57	AR-R2	*A. methanolica* NBRC 104435	96.51
AR-R3	*N. vanlangensis* TN01LGI	99.86	NKS-R1	*A. methanolica* NBRC 104435	99.92
KKS-R1	*N. vanlangensis* TN01LGI	99.85	NKS-RZ4	*A. methanolica* NBRC 104435	100.00
NKS-RZ3	*N. vanlangensis* TN01LGI	99.85	PS-RZ2	*A. methanolica* NBRC 104435	99.92
AR-RZ1	*N. vanlangensis* TN01LGI	99.93	KKS-RZ2	*A. methanolica* NBRC 104435	99.92
KKS-RZ4	*N. vanlangensis* TN01LGI	99.92	TBRC 15	*A. nitrogenifigens* TBRC 15 ^{T}	100.00
KKS-R3	*N. vanlangensis* TN01LGI	100.00	146AE	*B. cepacian* ATCC 25416	*
KKS-RZ3	*N. vanlangensis* TN01LGI	99.78	KKS-S1	*B. cepacian* ATCC 25416	98.91
TBRC 478	*G. sacchari* TBRC 478 ^{T}	100.00	KKS-R2	*B. cepacian* ATCC 25416	99.65
TBRC 480	*G. johannae* TBRC 480 ^{T}	100.00			

*: no identify by 16S rRNA
^{T}: Type strain

Primary screening

The cultures were grown in a Glucose-Yeast Extract-Peptone (GYP) medium (2% glucose (w/v), 1% peptone (w/v), 0.5% yeast extract (w/v). They were incubated at 30°C with shaking at 200 rpm for 24 h. Afterward, the concentration of the cultures was adjusted to 1 McFarland standard (Densitometer Densimat 99234, BIOMERIEUX ITALIA S.P.A., Italy), then they were grown again in the GYP medium, incubated at 30°C with shaking at 200 rpm for another 24 hours. Following this, the reactions were centrifuged

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at 8,000 rpm for 5 min. The clear portion (supernatant) was then analyzed using Thin Layer Chromatography (TLC).

Selection of thermotolerant AAB for 2-KGA production

The test strains were cultured in a liquid minimal medium using 1% sodium gluconate (w/v) as a substrate and were incubated at temperatures of 30 °C, 37 °C, and 40°C, shaking at 200 rpm for 24 hours. At the end of the incubation period, the cells were harvested by centrifugation at 8,000 rpm for 15 minutes. The quantity of fermentation products the acetic acid bacteria produced.

2-KGA production

The selected acetic bacteria are cultivated with sodium gluconate in a liquid minimal medium. This is because of the metabolic pathway that can directly lead to the production of 2-KGA. An initial concentration of 1% sodium gluconate (w/v) is used, and the fermentation is performed at a temperature of 40°C with agitation at 200 rpm for 24 hours., scaled up to a volume of 1 L. After fermentation, the reaction mixture is centrifuged at 8,000 rpm for 5 minutes, and the supernatant is collected for further analysis.

Determinations of 2-KGA

1. Thin layer chromatography (TLC)

The culture supernatant was spotted onto a TLC plate (Silica gel 60, Merck Co., Germany) with a volume of 10 µL of sample. The standard solutions of 2-KGA, 5-KGA, and glucose were compared. The plate was then immersed in a solvent system composed of ethyl acetate, acetic acid: methanol: distilled water in the ratios 6 : 1.5 : 1.5 : 1, respectively. After allowing it to dry, the plate was sprayed with a TTC solution (2% 2,3,5-triphenyl tetrazolium chloride (w/v) in a 0.5 M potassium hydroxide solution) or a freshly prepared color-developing reagent of aniline-diphenylamine phosphoric acid reagent (2% aniline (v/v), 2% diphenylamine (w/v) and 15% phosphoric acid (v/v) in acetone). The plate was then baked at a temperature of 120°C for 10-20 minutes. The intensity of the colors was compared with the standard solutions afterward.

2. Lanning and Cohen's method [16]

The product quantity was analyzed using Lanning and Cohen's method, which employs the o-Phenylenediamine dihydrochloride reagent (1.5% o-Phenylenediamine dihydrochloride (w/v) in 0.375 N HCl). When o-Phenylenediamine dihydrochloride reacts with 2-keto acids, it forms 2-hydroxyquinoxalines, forming a reddish-brown to green color. Subsequently, the absorbance was measured at 330 nm.

3. High-Performance Liquid Chromatography (HPLC)

Analysis was conducted to confirm the experimental results using HPLC with the HPLC Water e2695 Separations Module (Water Corporation 2013, USA). An Aminex® HPX-87H column, 300 mm x 7.8 mm (I.D. x Length), Bio-Rad) was utilized, and 5.0 mM Sulfuric Acid (H_2SO_4) was used as the mobile phase at a flow rate of 0.60 mL/min. The column temperature was maintained at 65°C. Detection was carried out using a Water 2998 Photodiode Array (PDA) Detector set at a wavelength of 210 nm. Results were interpreted by comparing them with the standard compounds of 2-KGA under the instrument conditions presented in Table 2.

Table 2 Analysis conditions for 2-KGA by HPLC.

Parameter	Optimized condition
Chromatograph:	HPLC Waters e2695 Separations Module
Column:	Aminex® HPX-87H (Bio-Rad)
Column Size:	300 mm x 7.8 mm (I.D. x Length)
Mobile phase:	5.0 mM Sulfuric Acid (H_2SO_4)
Flow rate:	0.60 mL /min
Detector:	Water 2998 Photodiode Array (PDA); 210nm
Column temp.:	65°C
Injection volume:	10 µL

Purification of 2-KGA via column chromatography

1. Ion-Exchange Chromatography

DEAE-Sephacel column chromatography: The 2-KGA obtained from acetic bacteria was purified using modified methods [17]. The initial step involves passing the sample through DEAE Sephacel column chromatography to eliminate undesired proteins and other impurities. The DEAE Sephacel gel was first activated for effective chromatography. This involved thoroughly rinseing with copious amounts of distilled water to remove 20% ethanol. Subsequently, the gel was immersed in 0.1 N NaOH for 30 minutes and then rinsed with large volumes of water until a neutral pH (7.0) was achieved. The gel was soaked in 0.1 N HCl for another 30 minutes and rinsed extensively to reach a neutral pH. Once ready, the gel was packed into a chromatography column. The column was then equilibrated by introducing twice the gel volume of 25 mM Tris-HCl (pH 8.0). The eluent was allowed to pass through the column until a pH of 8.0 was consistent in the effluent. The column was then connected to a fraction collector to separate the compounds. With a set flow rate of 1 mL/min, fractions were collected every 10 mL. A 450 mL sample was introduced into the column and washed with 2 column volumes of 25 mM Tris-HCl (pH 8.0). Once the entire sample had passed through the column, elution was carried out using 1 M NaCl, at three times the column volume. The protein profile of each fraction was monitored by measuring its absorbance at 280 nm, and compared against spots on filter paper sprayed with TTC. Fractions containing 2-KGA were pooled together and analyzed for the cumulative content of 2-KGA and proteins using Lanning

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and Cohen's method [16] and Lowry's method [18], respectively.

DOWEX 1X4 column chromatography: The sample from section DEAE-Sephacel column underwent further purification through DOWEX 1X4 column chromatography. The DOWEX 1X4 column was initially activated by neutralizing it with 0.1 N NaOH, followed by extensive washing with water until a neutral pH (7.0) was achieved. The gel was then equilibrated to the chloride form using 1 M $CaCl_2$. Subsequent steps involved the removal of Ca^{2+} ions by rinsing with copious amounts of water until no precipitation occurred upon adding the eluent to 0.5 M sodium bicarbonate. The gel was then packed into a chromatography column.100 mL of sample volume was loaded onto the column, followed by a wash with DI water at twice the volume of the column. After allowing all compounds to pass through the column, elution was conducted using 0.8 M and 1.0 M NaCl solutions at three times the column volume. Each fraction was monitored by spotting on filter paper and spraying with TTC, and further compared against reactions with o-Phenylenediamine dihydrochloride reagent [16]. Absorbance measurements were taken at a wavelength of 330 nm. Fractions containing 2-KGA were pooled together and subsequently analyzed for the total content of 2-KGA and proteins using Lanning and Cohen's [16] and Lowry's [18], respectively.

2. Gel Filtration Chromatography

Concentration of 2-KGA Solution: The clear fraction of the culture supernatant that has passed through the DOWEX 1X4 column was concentrated using rotary evaporation under conditions of low pressure at 35 mbar, a rotation speed of 50 rpm, and a temperature of 40°C until the sample volume was reduced tenfold (10x concentration).

Superdex™ S-200 Column Chromatography: The Superdex™ S-200 gel, which was stored in a 20% ethanol solution, was decanted. The gel was then washed with distilled water, stirred to ensure homogeneity, and allowed to settle. The clear supernatant was discarded. This washing procedure was repeated 2-3 times. The gel was then packed into an XK column (XK 16/100; I.D./Length, mm) following the instructions provided for Superdex™ S-200. The gel in the column was equilibrated with a 0.1 N NaCl solution at a flow rate of 60 mL per hour for 3 hours.

Loading sample: The concentrated 2-KGA solution, 0.5 mL, was loaded onto the Superdex™ S-200 column chromatography (as described in 2.) at a flow rate of 60 mL/h. This was followed by elution with a 0.1 N NaCl solution, collecting 1.5 mL/fractions. Each fraction's profile was monitored by spotting onto filter paper, spraying with TTC, and reacting with o-Phenylenediamine dihydrochloride before measuring the absorbance at a wavelength of 330 nm. Subsequently, fractions containing 2-KGA were pooled together to analyze 2-KGA and total protein using Lanning and Cohen's [16] and Lowry's [18]. The absorbance values were then measured at 330 and 490 nm wavelengths, respectively.

Color removal and precipitate removal

At a concentration of 1% (w/v), activated carbon powder was utilized to absorb the color from the 2-KGA product. The mixture was left undisturbed for at least 4 hours. to allow the charcoal powder to adsorb the color fully. Afterward, it was centrifuged to separate the charcoal powder sediment. The concentration of the product post-color adsorption was determined using Lanning and Cohen's method [16]. Subsequently, the sample was stored at a temperature of -80°C before being lyophilized with a freeze-dryer.

Total protein quantification [18]

The sample solution, with a volume of 0.4 mL, was added to a test tube. Lowry's solution A and Lowry's solution B were mixed in a ratio of 50 : 1 (A : B), and 2 mL of this mixture was added to the test tube. The contents were mixed thoroughly, and then incubated at 35°C for 10 min. Subsequently, 0.2 mL of 1 N Folin's reagent was added. Immediate mixing was done using a vortex mixer and then incubated at 35°C for 30 minutes. The absorbance of the solution was measured at 750 nm, and the results were compared against a BSA standard curve to determine the protein concentration in the sample.

RESULTS AND DISCUSSIONS

Primary screening of 2-KGA producer

The TLC technique was used to analyze the type of 2-KGA product derived from the 24-hour fermentation of 25 bacterial strains listed in Table 1. The experimental results revealed: 13 isolates, specifically AR-R2, TBRC478, TBRC479, NKS-RZ1, AR-R3, KKS-R1, TBRC480, NKS-RZ2, AR-RZ1, KKS-RZ4, TBRC4639, KKS-R3, and KKS-RZ3, could produce 2-KGA. This was evident as red spots emerged at positions consistent with the standard 2-KGA sugar solution on the culture medium. The isolated TBRC15 produced 5-KGA, indicated by the presence of an orange-red spot on the TLC plate that matched the position of the 5-KGA standard solution. Conversely, the substances produced by the isolates PS-RZ1, KKS-R4, KKS-RZ1, NKS-R1, NKS-RZ3, PS-RZ2, KKS-S1, 146AE, KKS-R2, KKS-RZ2, and AR-R1 in the culture medium were not the desired keto-sugars. They were found to be glucose, an aldose sugar group that did not produce a red spot when treated with TTC, as shown in Figure 1B. When sprayed with the color-developing reagent, aniline-diphenylamine phosphoric acid reagent, however, blue spots corresponding to the standard glucose solution appeared, alongside greenish-brown spots matching the 2-KGA standard and gray spots aligning with the 5-KGA standard, as shown in Figure 1A. When utilizing glucose, acetic acid bacteria's

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metabolic pathway involves the oxidation of D-glucose into D-gluconic acid. Subsequently, D-gluconic acid can be converted into either 2-KGA or 5-KGA through competitive reactions. Both 2-KGA and 5-KGA are then secreted out of the cell until the glucose is fully oxidized. Interestingly, certain strains of *Gluconobacter* are found to further oxidize 2-KGA into 2,5-DKGA via the enzyme 2-KGA dehydrogenase. This enzyme is membrane-bound and employs FAD as a cofactor [14]. Therefore, the results indicate that the isolates AR-R2, TBRC478, TBRC479, NKS-RZ1, AR-R3, KKS-R1, TBRC480, NKS-RZ2, AR-RZ1, KKS-RZ4, TBRC4639, KKS-R3, and KKS-RZ3 are of particular interest for further investigation into their ability to produce 2-KGA. These selected isolates were chosen for subsequent studies.

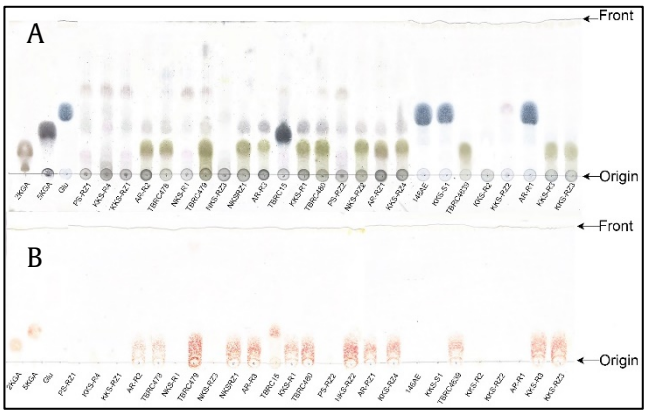

Figure 1 The oxidative products of 25bacterial strains after culture in 1% glucose (w/v) medium and detection by TLC technique using (A) aniline-diphenylamine phosphoric acid reagent and (B) TTC reagent.

Selection of thermotolerant AAB for 2-KGA production

Upon testing the growth of the 13 strains that could produce 2-KGA from the previous experiments, they were cultivated in a minimal liquid medium at temperatures of 30, 37, and 40°C to select strains that could grow at higher temperatures. The results showed that increasing temperatures had varying effects on the growth of the test organisms. All 13 isolates were found to grow well at 30°C. However, at 37°C, only 7 isolates, namely AR-R2, TBRC478, AR-R3, KKS-R1, AR-RZ1, KKS-R3, and KKS-RZ3, were able to grow effectively. Furthermore, at 40°C, only the isolates AR-R2, AR-R3, KKS-R1, AR-RZ1, KKS-R3, and KKS-RZ3 were found to grow in sequence. When analyzing the quantity of 2-KGA products using the chemical method by Lanning and Cohen [16]. The results revealed that at 30°C, all 13 isolates could produce 2-KGA. Among them, isolated TBRC479 displayed the highest production capacity, followed in order by isolates KKS-RZ3, AR-RZ1, KKS-R1, KKS-RZ3, NKS-RZ2, AR-R2, AR-R3, TBRC480, TBRC478, TBRC4639, KKS-RZ4, and NKS-RZ1. At 37°C, only the isolates KKS-R1, AR-RZ1, TBRC4639, AR-R3, AR-R2, KKS-R3, KKS-RZ3, TBRC478, and NKS-RZ2 were found to produce 2- KGA in that order. Meanwhile, at 40°C, only the isolates KKS-R1, AR-RZ1, AR-R3, KKS-RZ3, and AR-R2 showed 2-KGA production capabilities, with KKS-R 1distinctly showing robust consistency across all three tested temperatures, as represented in Figure 2. This evidence indicates that this group of AAB is thermotolerant. Typically, an increase in temperature can influence the enzymatic activity pivotal for converting substrates into products and impair the metabolic pathways of the microorganisms. Mesophilic acetic acid bacteria tend to thrive best between 25-30°C. In contrast, thermophilic strains can maintain good growth and metabolic activity at elevated temperatures, largely because the enzymes in these heat-resistant microorganisms naturally possess inherent thermostability [19]. Therefore, there has been an effort to select strains of AAB that can thrive at elevated temperatures, aiming to maximize utility while minimizing temperature-related limitations. This allows for broader applications in various industries that operate at higher temperatures. Consequently, the researcher is keen on further exploiting the *N. vanlangensis* KKS-R1 strain for enhanced 2-KGA production because it is most stable in production at higher temperature ranges.

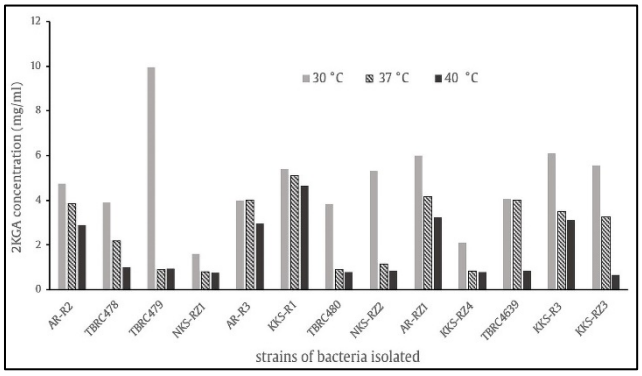

Figure 2 Production of 2-KGA from thirteen selection bacterial strains at 30, 37, and 40°C.

2-KGA production

From the experiment of producing 2-KGA using the *N. vanlangensis* KKS-R1 strain in a minimal liquid medium, with 1% gluconate (w/v) serving as the carbon source in a 1 L volume, and under shaking conditions at a rate of 200 rpm at a cultivation temperature of 37°C for 1 day, the total production amounted to 7.476 g/L.

Purification of 2-KGA

The culture supernatant (crude 2-KGA) was subjected to DEAE Sephacel column chromatography, characterized by its anionic properties. Each fraction was monitored by measuring the absorbance at a wavelength of 280 nm, a standard method for quantifying general protein levels. The results revealed two distinct peaks: peak 1 was located within the fraction range of 22-110, while peak 2 was identified in the fraction

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range of 111-142, as illustrated in Figure 3. These outcomes aligned with tracking via TTC, indicating the emergence of red spots solely within peak 1, fraction 22-100 (pool I), as demonstrated in (•). Conversely, peak 2, fraction 102-142 (pool II), showed an absence of these red spots. The fractions (pool I) with these evident red spots were then pooled together before undergoing 2-KGA quantification analysis using Lanning and Cohen's method [16]. Compared to the sample before column chromatography, the total 2-KGA yield was found to be 3.40±0.01 g, while the quantity prior to column passage was 3.74±0.04 g. Moreover, when the total protein quantity both before and after column passage was analyzed using Lowry's method [18], it was observed that pool I contained only 0.38±0.46 g of protein. This starkly contrasts the total protein measured before column chromatography, which was as high as 0.94±0.63 g. Some of the protein content was lost, with a significant 90.96 % of the desired compound eluted from the DEAE Sephacel column, as depicted in Table 3. This suggests that some proteins carrying positive charges were retained by the DEAE Sephacel column chromatography, which possesses anionic properties. These proteins were only eluted when washed with 1 M NaCl. In this phase, some of the proteins were separated, resulting in a purer desired compound.

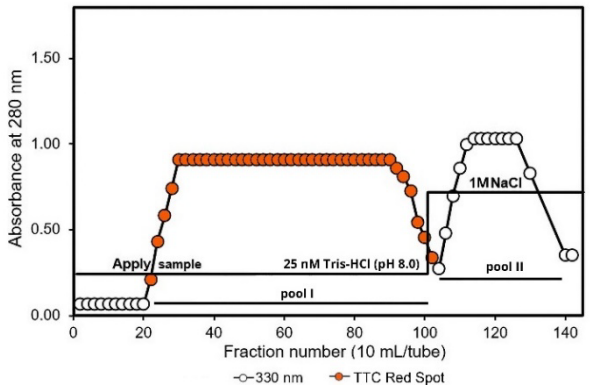

Figure 3 Chromatography of 2-KGA of *N. vanlangensis* KKS-R1 on DEAE Sephacel column. Culture supernatant was applied onto a DEAE Sephacel column previously equilibrated with 25 mM Tris-HCl buffer (pH 8.0). The elution of 2-KGA was made by 25 mM Tris-HCl buffer (pH 8.0) and eluted with 1 M NaCl.

After 2-KGA fractions from DEAE Sephacel column chromatography were pooled, they were loaded onto a DOWEX 1X4 column and adjusted to their chloride form, which binds to positively charged compounds. Tracking the 2-KGA profile by spotting on filter paper and spraying with TTC reagent showed slight red spots in fractions 12-26 (pool I) during the sample loading and wash phases (fractions 1-58), as red spots indicated (•). As the elution phase (fractions 59-98) using 8.0 M NaCl three times the volume began, red spots appeared from fractions 60-96 (pool II) as red spots indicated (•), with no color observed in other fractions. Pooling and analyzing these fractions showed peaks consistent with the TTC test results. The wash phase peak might arise from neutrally charged compounds having weak column binding, resulting in some desired compound release after water wash. All compounds were completely eluted during the elution step, presenting as the second peak, with no substances observed post-elution with 1 M NaCl, as depicted in Figure 4. Upon analyzing the total 2-KGA quantity in comparison to pre-DOWEX 1X4 column values, the post-column product amounted to 3.40±0.01 g, and the pre-column content was 3.74±0.04 g. This signifies that purification using the DOWEX 1X4 column post. protein separation via DEAE Sephacel column chromatography improved purity, yielding up to 75% of the desired compound. However, protein analysis revealed a residual protein content of 0.06±0.002 mg/mL in the product, as shown in Table 3. Consequently, this product would undergo further purification stages.

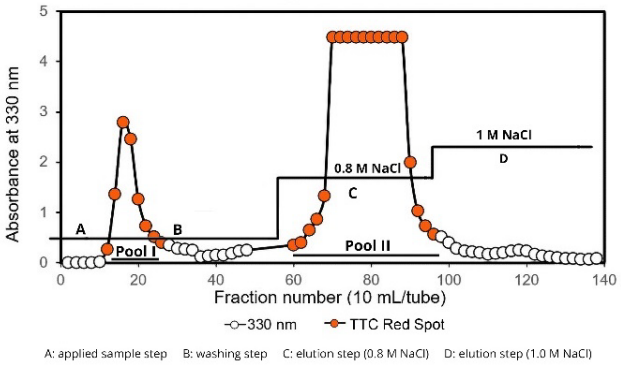

Figure 4 Elution profile of 2-KGA from pooled I of DEAE Sephacel column applied onto DOWEX 1X4 column chromatography previously equilibrated with 1 M $CaCl_2$ under the conditions described in Materials and Methods. Elution was done with a stepwise of 0.8 M NaCl and 1 M NaCl as indicated. (A: applied sample step, B: washing step, C: elution step (0.8 M NaCl) and D: elution step (1.0 M NaCl)).

The 2-KGA sample (pool II), primarily obtained from purification via the DOWEX 1X4 column, was purified using the Superdex™ S-200 column chromatography. The profile of the eluted components was monitored using Lanning and Cohen's method [16]. Experimental results showed that the profile of substances eluted from the column had only one peak, spanning fractions 94 to 108 (pool I). This result correlates with the TTC test, where red spot appeared, as indicated in (•). Furthermore, when monitoring the protein content in each fraction by measuring the absorbance at a wavelength of 280 nm, a protein peak was observed in fractions 18 to 86. No peaks were evident in fractions 94 to 108, suggesting that the Superdex™ S-200 column chromatography effectively eliminated the proteins. Consequently, when pooling the fractions from 94 to

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108, the overall yield of 2-KGA was determined to be 2.654 g, resulting in a yield of 71.02%. Meanwhile, Lowry's method reduced the overall protein concentration to undetectable levels [18] (Table 3).

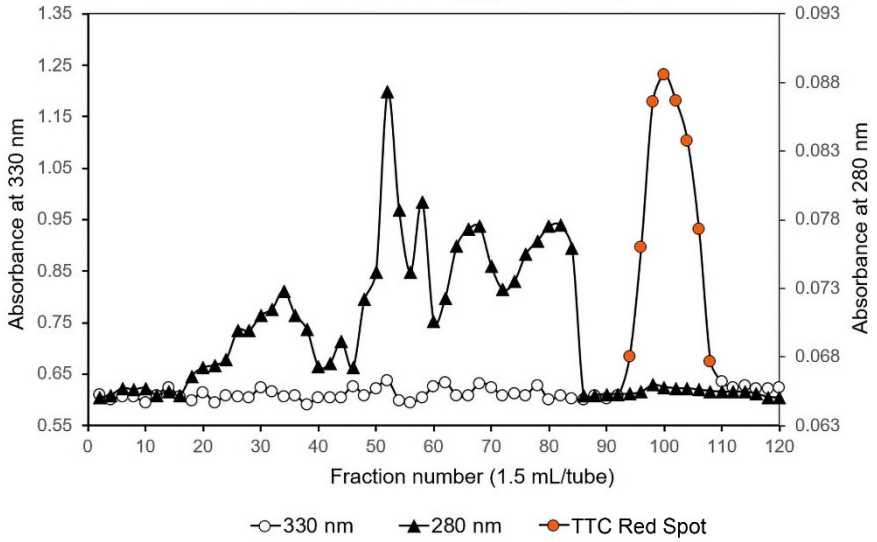

Figure 5 Gel filtration profile of 2-KGA from *N. vanlangensis* KKS-R1. The 2-KGA was obtained from pooled II of DOWEX 1X4 column chromatography was loaded onto Superdex™ column chromatography (Ø1.6 cm x 90 cm) equilibrated with 0.1 N NaCl and eluted at a flow rate of 22 mL/h. Absorbance is at 330 nm (open circles) and 280 nm (closed triangles).

Table 3 Purification summary of 2-KGA from *N. vanlangensis* KKS-R1.

Step of Purification	Total Volume (mL)	2-KGA Content (mg/mL)	Total 2-KGA (g)	Protein content (mg/mL)	Total Protein (g)	%yield of 2-KGA
Crude 2-KGA	500.00	7.47±0.04	3.74±0.04	1.88± 0.07	0.94± 0.63	100.00
DEAE Sephacel	630.00	5.40±0.01	3.40±0.01	0.60±0.01	0.38±0.46	90.96
DOWEX 1X4 (Pool I)	62.00	0.27±0.01	0.17±0.04	0.08±0.002	0.01±0.004	4.90
DOWEX 1X4 (Pool II)	350.00	8.08±0.02	2.96±0.33	0.06±0.002	0.02±0.73	75.00
Superdex S-200	35.00	75.84±0.016	2.654±0.18	ND*	ND*	71.02

*ND: Not detected

Figure 6 The Characteristics of 2-KGA powder after activated carbon adsorption and crystallization by freeze-drying.

Decolorization and precipitation

The decolorization of the 2-KGA product obtained from the experiment was performed using activated charcoal, known for its decolorizing properties. The mechanism behind this decolorization process involves weakly binding through van der Waals forces between organic compounds and the external surface of the charcoal [20]. When the charcoal was added to the pale yellow 2-KGA solution and allowed to settle for 4 hours, the solution's color became clearer. Before undergoing freeze-drying, the decolorized solution turned into a white powder, as shown in Figure 6.

Analysis of purity of the 2-KGA product using HPLC and TLC

After subjecting the powder solution of the purified 2-KGA product, obtained from the separation through the three types of columns, to analysis using High-Performance Liquid Chromatography (HPLC), it was observed that two distinct peaks emerged. The main peak exhibited a retention Time value of 9.857 minutes, corresponding to the retention time of the standard 2-KGA solution (data not shown). The secondary peak displayed a Retention Time value of 7.529 minutes, aligning with the Retention Time of the standard NaCl solution (data not shown). This is illustrated in Figure 7A. Furthermore, when tested using Thin-Layer Chromatography (TLC) and sprayed with 2,3,5-Triphenyltetrazolium chloride (TTC), only a single spot corresponding to the standard 2-KGA solution was observed, as depicted in Figure 7B.

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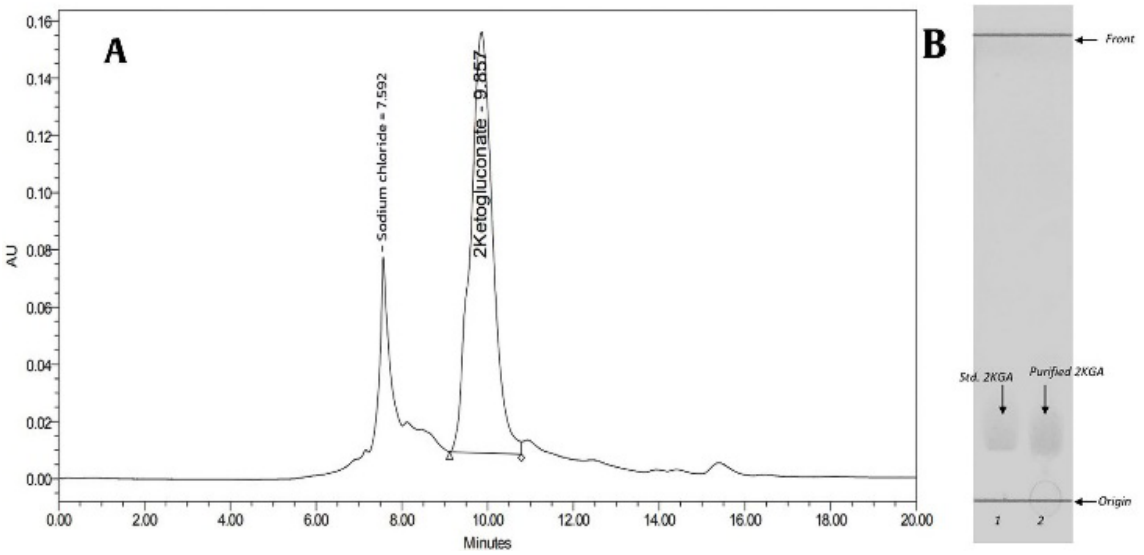

Figure 7 Purity analysis of 2-KGA product obtained from *N. vanlangensis* KKS-R1 by HPLC (A) and TLC (B).

CONCLUSION

The acetic acid bacterium *Nguyenibacter vanlangensis* KKS-R1 has been identified as exceptionally capable of producing 2-KGA at high temperatures up to 40°C, utilizing a minimal liquid medium with sodium gluconate as the carbon source. When the produced 2-KGA is further purified through ion exchange chromatography, two types of columns, a DEAE Sephacel column chromatography, which functions as an anion, and a DOWEX 1X4 column in its chloride form, before being subjected to gel filtration chromatography for increased purity, this efficient process allows the isolation of highly pure 2-KGA with a concentration of 81.90±0.03 mg/mL, constituting 71.02% of the total yield. Subsequently, the 2-KGA product undergoes a decolorization process using activated carbon and is then freeze-dried, resulting in a pure white powder. This study suggests that these processes are viable for industrial application and can be further optimized to enhance production efficiency.

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