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A plate method for rapid screening of *Ketogulonicigenium vulgare* mutants for enhanced 2-keto-L-gulonic acid production

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ABSTRACT

A new plate method was developed for rapid screening of *Ketogulonicigenium vulgare* mutants overproducing 2-keto-L-gulonic acid (2-KLG). The screening methodology took the advantage of the acidity caused by 2-KLG, which changes the color of bromothymol blue (pH indicator) from blue to yellow. Using the proposed method, a mutant, *K. vulgare* 65, was selected from 20,000 colonies produced by a strain subjected to spaceflight mutagenesis. When co-cultured with *Bacillus megaterium* 2980 in 20-L fermenters, *K. vulgare* 65 showed a high conversion rate (94.45%) of L-sorbose to 2-KLG. In contrast to the traditional screening method, this one significantly improved the frequency of obtaining positive mutants. The proposed plate screening method is cost-effective and easy to run and is thus useful for the isolation and screening of *K. vulgare* mutants overproducing 2-KLG.

Introduction

2-Keto-L-gulonic acid (2-KLG) is an important precursor for the synthesis of vitamin C (L-ascorbic acid) by a two-step fermentation process, which accounts for 90% of the global vitamin C production.[1] Research has shown that 2-KLG can be bioconverted from L-sorbose by a mixed culture of *Ketogulonicigenium vulgare* and *Bacillus* spp.[2,3] *K. vulgare* contains a complete enzyme system for converting L-sorbose to 2-KLG,[4] but its growth is poor in the monoculture, resulting in a very low yield of 2-KLG.[5] A companion strain, *Bacillus megaterium*,

is generally considered to generate and release, in the fermentation broth, the metabolites that stimulate *K. vulgare* propagation and 2-KLG accumulation.[6–10] These metabolites contain active proteins,[6] amino acids,[8] antioxidants,[10] and other active substrates.[7,9] In recent years, *K. vulgare* has been considered to be the key microorganism for the bioconversion of L-sorbose to 2-KLG and of greater importance than the companion bacterium in 2-KLG production.[11,12]

Random mutagenesis and selection strategies are effective for the screening of *K. vulgare* mutants capable of 2-KLG overproduction.[13,14] When obtaining high-efficiency mutants of *K. vulgare*, it is crucial to screen a large number of mutants

immediately after a mutagenic treatment. Thus, easy and efficient screening methods are desired for the screening and isolation of 2-KLG-overproducing mutants of *K. vulgare*. Since mixed fermentation involves co-cultivation of two bacterial strains, the associated screening process is relatively laborious and complicated.[7] Currently, only one method is available for the screening of *K. vulgare* mutants, i.e., the 2-KLG determination method.[13] In this method, the primary screening is done by randomly selecting *K. vulgare* colonies on plates. Then, the colonies are used for fermentation with *Bacillus* spp. in glass tubes, followed by the determination of the amount of 2-KLG produced. After that, *K. vulgare* mutants with high yields of 2-KLG are selected and transferred to flasks for further fermentation. Finally, *K. vulgare* mutants capable of highly efficient conversion of L-sorbose to 2-KLG are selected as potential strains for industrial applications. As it involves random colony selection in the primary screening step, followed by laborious in-tube fermentation and determination of 2-KLG concentrations, this conventional screening method is considered inefficient and time-consuming.

In the present study, a new plate screening method was developed for screening of *K. vulgare* mutants with a high conversion rate of L-sorbose to 2-KLG. The proposed method is based on the following points: (1) the active metabolites (activators) secreted by *B. megaterium* enhance the growth of *K. vulgare*[5,6,8–10]; and (2) the 2-KLG secreted by *K. vulgare* mutants lowers the medium pH in a certain zone on agar plates, thereby changing the color of the bromothymol blue indicator from blue to yellow. The proposed method was designed for rapid screening of *K. vulgare* mutants to detect those capable of 2-KLG overproduction. It was further applied to the screening of 20,000 colonies of *K. vulgare* obtained after spaceflight mutagenesis, among which the mutant *K. vulgare* 65 was selected. *K. vulgare* 65 was co-cultured with *B. megaterium* under laboratory conditions, and its conversion rate of L-sorbose to 2-KLG was monitored. The results were used to evaluate the efficiency of the indicator plate method for the isolation and screening of 2-KLG-overproducing mutants of *K. vulgare*.

Materials and methods

Bacterial strains

The candidate 2-KLG-producing strains, *K. vulgare* 07 and *B. megaterium* 2980, were obtained from Northeast Pharmaceutical Group Co., Ltd. (Shenyang, China).

Laboratory media

Three media were prepared for the maintenance, growth test, and isolation, respectively, of *K. vulgare* 07 and *B. megaterium* 2980. These included L-sorbose-limiting medium (20.0 g of L-sorbose, 3.0 g of corn steep liquor, 1.0 g of carbamide, 1.0 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 1.0 g of $CaCO_3$ per liter; pH 6.7–7.0), fermentation medium (80.0 g of L-sorbose, 15.0 g of corn steep liquor, 12.0 g of carbamide, 1.0 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 1.0 g of $CaCO_3$ per liter; pH 6.7–7.0;

L-sorbose and carbamide were sterilized separately), and isolation medium (20.0 g of L-sorbose, 5.0 g of yeast extract, 5.0 g of corn steep liquor, 10.0 g of peptone, 3.0 g of beef extract, 1.0 g of carbamide, 1.0 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 18 g of agar per liter; pH 7.0–7.2). The media were autoclaved prior to use.

Spaceflight mutagenesis

Spaceflight mutagenesis has been shown to be more efficient than other mutation methods for microbes.[15–17] The unique environments in the outer space, such as microgravity, strong radiation, the presence of high-energy particles, and alternating magnetic fields, can induce gene mutations.[18] In this study, the spaceflight mutagenesis technology was used to obtain more mutants.

The two candidate strains, *K. vulgare* 07 and *B. megaterium* 2980, were co-cultured on L-sorbose-limiting medium in Eppendorf tubes for 48 h and then transferred to the returning module of the manned spacecraft Shenzhou VIII. The spacecraft was launched at 5:58 a.m. (Beijing time) on November 1, 2011 from the Jiuquan Satellite Launching Center (Gansu, China). After a 397-h flight in outer space, the returning module landed in the Inner Mongolia Autonomous Region, China. The Eppendorf tubes with an intact package were kept in an icebox and transported immediately to the laboratory for use.

Effects of B. megaterium-secreted activators on the growth of K. vulgare

The culture of *K. vulgare* 07 was prepared as 10-fold serial dilutions. A 0.1-mL aliquot of the diluted culture, containing 30–50 colony-forming units (CFU), was spread on an agar plate containing 30 mL of solidified isolation medium. Two agar plates were inoculated. To one plate, 0.1 mL of co-culture broth of *B. megaterium* 2980 and *K. vulgare* 07 (30-h fermentation at 29 °C, $OD_{650} = 0.935$), pre-filtered through a sterile 0.22-μm membrane filter, was added and spread. The other agar plate was used as a control. The two plates were incubated at 29 °C for 96 h. The diameters of 40 colonies on each plate were determined using a Vernier caliper (0–150, Harbin Measuring & Cutting Tool Group Co., Ltd., Heilongjiang, China).

Choice of pH indicator

Three pH indicators were tested for the direct determination of 2-KLG production on plates, including methyl red [2-(4-dimethylaminophenylazo)benzoic acid; Tianjin Yongda Chemical Reagent Co., Ltd., Tianjin, China], bromocresol purple (5,5′-dibromo-O-cresolsulfonphthalein; Shenyang Xinxi Reagent Factory, Liaoning, China), and bromothymol blue (3′,3″-dibromothymolsulfonphthalein; Tianjin Bodi Chemicals Co., Ltd., Tianjin, China). Each indicator was dissolved in distilled water containing 5% (v/v) ethanol, and the concentration was adjusted to 0.1% (w/v). One milliliter of each solution was added dropwise to each agar plate to indicate the color changes around the colonies of *K. vulgare*. The indicator that showed an obvious color change was chosen as a suitable pH indicator.

New plate screening method

The schematic of the plate screening procedure is shown in Fig. 1. First, 10-fold serial dilutions of a culture of *K. vulgare* 07 subjected to spaceflight mutagenesis were prepared. A 0.1-mL aliquot of the diluted culture containing 30–50 CFU/mL was then spread on an agar plate containing 30 mL of solidified isolation medium. Second, 0.1 mL of co-culture broth of *B. megaterium* 2980 and *K. vulgare* 07 (30-h fermentation at 29 °C, $OD_{650} = 0.800$–1.000), pre-filtered through a sterile 0.22-μm membrane filter, was added to each agar plate inoculated with the spaceflight-induced *K. vulgare* mutants. After 96 h of incubation at 29 °C, colonies of *K. vulgare* mutants appeared on each plate. Third, to obtain pure mutant isolates, cells from each colony grown on agar plates were transferred to fresh agar plates using velvets and incubated at 29 °C for 96 h. Finally, 1 mL of a bromothymol blue solution (0.1%,

w/v) was added dropwise to each agar plate, and the plates were incubated at 29 °C for 30–60 min. Yellow zones were observed around the colonies of *K. vulgare* 07 mutants, and the diameters of the yellow zones were measured using a Vernier caliper. Colonies with the largest diameters of yellow zones on agar plates were selected as potential mutants of interest for further flask fermentation screening. Approximately 20,000 colonies of *K. vulgare* 07 were screened, and 500 potential mutants were selected for further screening.

Shake-flask fermentation screening and fermentation tests

After the primary agar plate screening, the selected mutants were further screened by shake-flask fermentation. The *K. vulgare* 07 mutants were co-cultured with *B. megaterium* 2980 in triplicate 250-mL Erlenmeyer flasks containing 20 mL of fermentation medium. After 48 h of fermentation at 29 °C on a
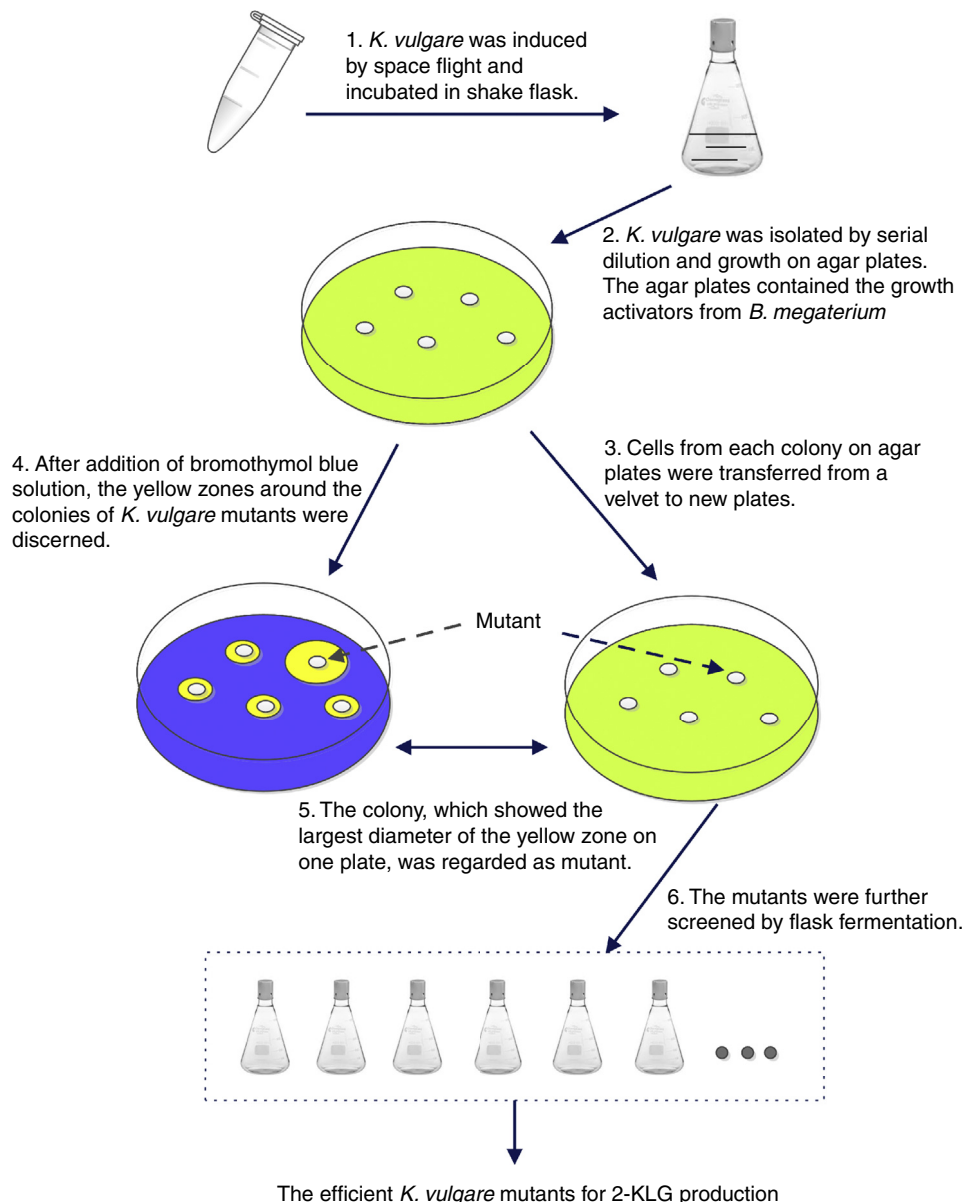

1. *K. vulgare* was induced by space flight and incubated in shake flask.

2. *K. vulgare* was isolated by serial dilution and growth on agar plates. The agar plates contained the growth activators from *B. megaterium*

4. After addition of bromothymol blue solution, the yellow zones around the colonies of *K. vulgare* mutants were discerned.

3. Cells from each colony on agar plates were transferred from a velvet to new plates.

Mutant

5. The colony, which showed the largest diameter of the yellow zone on one plate, was regarded as mutant.

6. The mutants were further screened by flask fermentation.

The efficient *K. vulgare* mutants for 2-KLG production

Fig. 1 – The procedure of screening of mutated strains by the pH indicator method.

rotating platform shaker (HZQ-R, Donglian Group Co., Ltd., Heilongjiang, China) at 220 rpm, a *K. vulgare* 07 mutant with the highest production of 2-KLG (*K. vulgare* 65) was chosen to evaluate the fermentation stability in 20-L fermenters. The 2-KLG concentration in the fermentation broth was determined by iodometry.[19] The sorbose concentration was measured by the sulfuric anthrone reaction and detected at 620 nm using a UV–Vis spectrophotometer (VIS-7220, Rayleigh Analytical Instrument Co., Ltd., Beijing, China).[20] The conversion rate was defined as the ratio of the 2-KLG yield in the fermentation broth to a theoretical value.

Comparison of traditional and pH indicator screening methods

The culture of *K. vulgare* 07 subjected to spaceflight mutagenesis was prepared as 10-fold serial dilutions and spread on agar plates. For the traditional screening method, colonies of *K. vulgare* were selected randomly, then inoculated with *B. megaterium* 2980 in 250-mL flasks containing 20 mL of the fermentation medium, and incubated at 29 °C for 38 h.[21] The colonies that produced higher concentrations of 2-KLG (≥62.0 mg/mL) were regarded as positive mutants. For the new screening method, the agar-plate colonies with the largest diameters of zones whose color changed after the addition of 1 mL of a bromothymol blue solution (0.1%, w/v) were selected for further flask fermentation screening, similar to the traditional screening method. Fifty colonies were chosen from agar plates by each screening method for flask fermentation. The number of positive mutants obtained by each method was counted.

Statistical analysis

The difference in 2-KLG production between groups was evaluated using the Student's *t*-test in the Statistical Program for Social Sciences (SPSS 13.0 for Windows, SPSS, Inc., Chicago, IL, USA). A *p*-value of <0.05 was considered statistically significant.

Results

Effects of B. megaterium filtrate on growth of K. vulgare

The results of the bacterial growth test showed that the average colony diameter (4.209 mm) of *K. vulgare* 07 incubated on a plate with a *B. megaterium* 2980 filtrate was significantly greater (*p* < 0.05) than that (3.019 mm) of *K. vulgare* 07 incubated without the *B. megaterium* 2980 filtrate. A further growth test of *K. vulgare* 07 with gradient volumes (0.1, 0.2, and 0.3 mL) of the *B. megaterium* 2980 filtrate showed no statistically significant differences (*p* > 0.05) in the colony diameters (data not shown). These observations were consistent with reports showing that *B. megaterium* acts as a companion bacterium and secrets one or multiple activators promoting the growth of *K. vulgare*.[5,22] Because of the increased cell number of *K. vulgare* 07, the corresponding 2-KLG concentration was improved in the fermentation broth.[21] Together, these findings indicate that the

activators secreted by *B. megaterium* increase the growth rate of *K. vulgare* 07, enhancing the production of 2-KLG.

Choice of pH indicator

When selecting bacterial mutants with enhanced 2-KLG productivity, simple and reliable methods are desired for the qualitative analysis of 2-KLG production. Here, we developed a plate method for the screening and isolation of *K. vulgare* mutants with altered 2-KLG productivity. The rationale was based on the fact that the 2-KLG secreted by colonies of *K. vulgare* 07 lowers the surrounding pH on an agar plate. To this end, a proper indicator is needed to show the changes in the pH of the medium. Upon the addition of a pH indicator, the color of the zone around a mutant colony grown on an agar plate would change. Theoretically, the diameter of the zone with a changed color should increase with the increase in the production of 2-KLG. In this study, three pH indicators were tested for the direct determination of 2-KLG production on a plate. The results showed that bromothymol blue was superior to the other two indicators in terms of the obvious color change and a suitable pH range (blue to yellow at pH 6.5–7.5). Upon the addition of a bromothymol blue solution (0.1%, w/v) to an agar plate, zones surrounding colonies of *K. vulgare* 07 mutants changed their color from blue to yellow because *K. vulgare* 07 mutants released 2-KLG on agar plates, thereby acidifying surrounding areas around colonies.

Relationship between the size of color-changing zone and 2-KLG concentration

When a given volume of a 2-KLG solution was pipetted onto an agar plate, yellow zones were observed. To evaluate the correlation between the size of the yellow color zone and the concentration of the bacterially produced 2-KLG, 50 μL of a 2-KLG solution (0–20 g/L) was added to an agar plate. With an increasing dose of 2-KLG, the yellow zones accordingly increased (Fig. 2). The correlation analysis showed that the diameter of the color-changing zone was linearly correlated ($R^2 = 0.984$) with the quantity of 2-KLG released into the agar.

Newly proposed agar plate screening method

Based on the above experimental results, a simple and rapid agar plate screening method was developed for the preliminary screening and isolation of *K. vulgare* mutants capable of efficient production of 2-KLG. The schematic of the plate screening procedure is shown in Fig. 1, and the detailed description of the new method is provided in Materials and Methods.

Comparison of conventional and pH indicator screening methods

The colonies of *K. vulgare* 07 grown after spaceflight mutagenesis were screened by the two different screening methods to compare their screening efficiencies. The results showed that by using the traditional screening method, only three positive mutants were obtained after screening 50 colonies randomly selected from 50 agar plates each (about 2000 colonies in total).
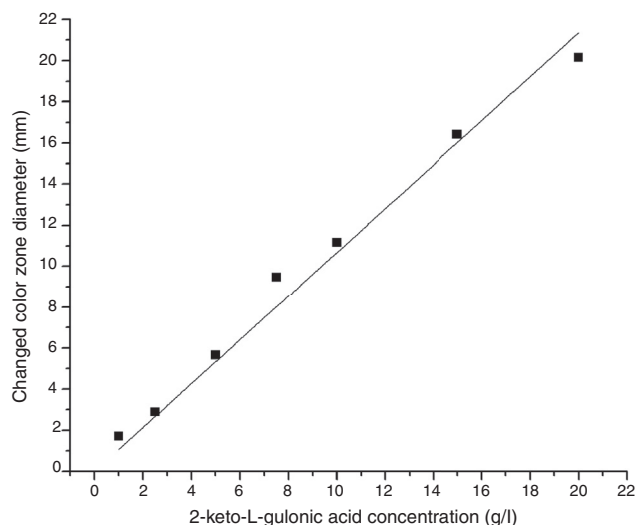
Fig. 2 – Relationship between 2-keto-ʟ-gulonic acid concentration and the change in the diameter of color zone of bromothymol blue pH indicator on agar plates.

On the contrary, by using the new screening method, 19 positive mutants were obtained after screening 50 colonies from 50 agar plates each. The results indicated that the new screening method greatly improved the probability of obtaining positive mutants.

Screening of K. vulgare mutants using proposed pH indicator plate method

In order to verify the efficiency of the newly established pH indicator plate method, we screened more than 20,000 post-spaceflight mutagenesis colonies of *K. vulgare* 07. After agar plate screening and flask fermentation screening, nine efficient mutants were finally obtained. Each of these mutants displayed a significantly higher ($p < 0.05$) 2-KLG yield than that of the parent strain (Table 1). The highest 2-KLG concentration (73.75 g/L) was achieved in a co-culture of *K. vugare* 65 and *B. megaterium* 2980, with the rate increase of 12.75% relative to that in a co-culture of the parent strain (*K. vulgare* 07)

Table 1 – Determination of 2-KLG produced by screened mutants in flask fermentation.

Screened mutants	2-KLG (g L^{-1})
KB06-2980	72.54 ± 0.16[a]
KB10-2980	68.10 ± 0.29[a]
KB21-2980	71.44 ± 0.22[a]
KB22-2980	69.83 ± 0.30[a]
KB23-2980	72.42 ± 0.71[a]
KB27-2980	70.40 ± 0.25[a]
KB37-2980	70.10 ± 0.56[a]
KB38-2980	70.52 ± 0.28[a]
KB65-2980	73.75 ± 0.24[a]
KB07-2980 (parent strains)	65.04 ± 0.44

All the data are presented as mean ± S.D.
[a] Statistically significant compared with parent strains.

Table 2 – Fermentation of KB65-2980 in 20 L fermenters for 10 batches.

	KB65-2980 (mutant strains)	KB07-2980 (parent strains)
2-KLG (g L^{-1})	91.63 ± 1.40[a]	84.74 ± 1.38
Conversion rate (%)	94.45 ± 1.44[a]	87.34 ± 1.42
Period (h)	41.0 ± 2.7[a]	44.0 ± 2.8
Productivity (g L^{-1} h^{-1})	2.24 ± 0.13[a]	1.93 ± 0.13

All the data are presented as mean ± S.D.
[a] Statistically significant compared with parent strains.

and *B. megaterium* 2980. Furthermore, an average ʟ-sorbose-to-2-KLG conversion rate of 94.45% and 2-KLG productivity of 2.24 g L^{-1} h^{-1} were achieved by co-cultivation of *K. vulgare* 65 and *B. megaterium* 2980 (KB65-2980) in 10 batches in 20-L fermenters (Table 2).

Discussion

Despite the rapid development of novel techniques,[23] conventional methods such as mutagenesis and selection based on direct measurements still play a central role as reliable and cost-effective procedures for strain improvement.[13,22,24] However, the need to use a mixed fermentation system for 2-KLG production by *K. vulgare* and *B. megaterium* makes traditional screening methods for *K. vulgare* mutants capable of 2-KLG overproduction laborious and complicated,[7] leading to a much lower screening efficiency.

In the present study, we described a rapid screening procedure for high-efficiency 2-KLG-overproducing mutants of *K. vulgare*. The proposed method avoids the negative effects of the active companion bacterium on the growth and conversion activities of *K. vulgare*, thus significantly increasing the probability of obtaining highly efficient *K. vulgare* mutants. The new method is also advantageous because of the ease of operation and the possibility of screening a large number (thousands) of mutants by one operator within a short period (<30 days). Compared with the conventional screening method, our proposed pH indicator plate method shortens the screening time by 90% and needs no large instruments.

Despite that the new method allows screening of a large number of *K. vulgare* mutants in the preliminary screening step much faster, the selected mutants should be further confirmed by co-fermentation with *B. megaterium* for the following two reasons. On one hand, the indicator method used in this study cannot differentiate between secretion of 2-KLG and other acidic metabolites by *K. vulgare* mutants, which may influence the accuracy of the plate screening results. On the other hand, more importantly, industrial production of 2-KLG is carried out in a mixed fermentation mode; hence, the 2-KLG production efficiency of *K. vulgare* mutants must be tested in a co-culture with *B. megaterium*.

The screening method provides an approach to screening for efficient mutants for other mixed fermentation systems. With a slight adjustment of culture conditions and selection of an appropriate pH indicator, the proposed pH indicator plate method can easily be used for the screening of other

bacterial strains suitable for the production of acidic products in a mixed fermentation system.

Conclusions

In this study, we developed a novel pH indicator plate method for the isolation and screening of *K. vulgare* mutants capable of overproduction of 2-KLG. Compared with the existing conventional method, the proposed screening method is more rapid, sensitive, and reliable. In addition, it is cost-effective and easy to use.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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