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Comprehensive Quality Evaluation of Corn Steep Liquor in 2-Keto-L-gulonic Acid Fermentation

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Supporting Information

ABSTRACT: Corn steep liquor (CSL) is one of the main raw materials in 2-keto-L-gulonic acid (2-KLG) fermentation by *Ketogulonicigenium vulgare* and *Bacillus megaterium*. Due to its natural origin and variations in the manufacturing process, unpredicted and uncontrolled variability of CSL has a great influence on 2-KLG production; however, conventional quality specifications are not enough to ensure stability of fermentation behaviors. A process analytical technology (PAT) could be considered to explore the relationship between CSL quality and 2-KLG production comprehensively. The compositions of CSL from six manufacturers were profiled by gas chromatography with time-of-flight mass spectrometry (GC-TOFMS) and inductively coupled plasma-atomic emission spectroscopy (ICP-AES), combined with orthogonal partial least-squares discriminant analysis (OPLS-DA). Seventeen components were identified as the most discriminant marker compounds related to 2-KLG production. Results revealed that they were responsible for providing nutrients and protecting osmotic pressure. Furthermore, nine amino acids were verified as potential group markers by addition to the medium and demonstration of the correlation to 2-KLG production. The comprehensive approach provided an important platform to explore CSL marker compounds for quality evaluation in 2-KLG fermentation.

KEYWORDS: corn steep liquor, quality evaluation, marker compound, PAT, 2-KLG fermentation

INTRODUCTION

2-Keto-L-gulonic acid (2-KLG), the precursor of vitamin C, is synthesized by *Ketogulonicigenium vulgare* and *Bacillus megaterium*. Due to a lack of various biosynthetic pathways of *K. vulgare*, it requires rich nutrients provided by sorbose−corn steep liquor (CSL) medium. CSL is a byproduct of the corn wet-milling industry, containing amino acids, minerals, vitamins, carbohydrates, organic acids, enzymes, and other elemental nutrients.[1] However, the CSLs (substrates) that give higher productivities are subjected to uncontrolled variability in compositions owing to different geographic origins of corn, different harvesting times, and different manufacturing processes, and therefore different batches of CSL reveal different behaviors.[2] At present, most of the available CSLs on the market are standardized for conventional quality control parameters, such as dry weight (DW, >40%), crude protein (>40%), sulfite (<0.3%), and acidity (<14%).[3] However, some compounds should also be taken into consideration. Recently, six compounds, glycine, serine, biotin, proline, nicotinic acid, and threonine, in the CSL powder have been demonstrated to be related to the 2-KLG fermentation process.[4] Also, Zhou et al.[5] reported that metal elements play an important physiological role in cell growth and 2-KLG accumulation. To date, most studies have focused on the composition determination of CSL. Amino acids and vitamins were analyzed by high-performance liquid chromatography (HPLC).[4,6] Hull et al.[7] reported that gas chromatography−mass spectrometry (GC-MS) was used to determine carbohydrates, lactic acid, glycolic acids, and fatty acids of corn steep water during steeping. Metal elements were detected by atomic absorption spectrometry (AAS) and inductively coupled plasma-atomic emission spectroscopy (ICP-AES).[4,7] Despite all of these attempts, no reports on the relationship between CSL quality and 2-KLG production have been published so far.

Currently, marker compounds, used for quality control of products from natural botanical sources, were proposed by the European Medicines Agency (EMEA).[8] Because conventional quality specifications are not enough to ensure stability of fermentation behaviors, selecting adequate and suitable marker compounds for CSL would be required. A process analytical technology (PAT) is expected to make a significant contribution to improving the process, because it is an efficient and creative approach for the quality control of raw materials in pharmaceutical manufacturing processes.[9] Although this strategy is applied in the first step of the process (raw material analysis), it is very necessary for manufacturing processes to reduce waste, improve productivity, and ensure high quality of the final products. In particular, high-throughput and high-resolution gas−liquid chromatography with mass spectral detection (GC-MS) and ICP-AES are robust tools used in a PAT for qualitative and quantitative analysis raw materials.[10−12] The multivariate statistical analysis technique became the other leg of PAT to obtain information from these overwhelming data. Principal component analysis (PCA),[13] hierarchical clustering analysis (HCA),[14] and orthogonal partial least-squares discriminant analysis (OPLS-DA)[15] were successfully employed in research for marker

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Table 1. Conventional Quality Parameters of Six Different CSL Samples (All Parameters Converted to Dry Weight by Calculation)

no.	manufacturer	appearance	dry weight (%)	crude protein[a] (%)	sulfite (%)	acidity[b] (%)	ash (%)
1	Jiangsu Xinyi Henghui Starch Sugar Co., Ltd., China	tawny, thick (+)	45.38 ± 0.13	49.67 ± 0.41	0.16 ± 0.03	7.8 ± 0.1	16.43 ± 0.28
2	Henan Julong Starch Industrial Co., Ltd., China	brown, thick (+)	45.04 ± 0.99	46.41 ± 1.42	0.18 ± 0.02	12.8 ± 0.3	16.41 ± 0.35
3	Kangxin of North China Pharmaceutical Co., Ltd., China	brown, thick (+)	45.99 ± 0.23	49.94 ± 0.75	0.11 ± 0.01	11.1 ± 0.8	15.49 ± 0.09
4	Zhongrun Pharmaceutical of Shijiazhuang Co., Ltd., China	yellow, thick (++)	47.00 ± 0.17	42.98 ± 0.55	0.18 ± 0.02	11.1 ± 0.7	12.97 ± 3.36
5	Tianjin Chemical Distribution Division Co., Ltd., China	brown, thick (−)	33.21 ± 0.96	51.84 ± 2.38	0.22 ± 0.01	11.1 ± 0.7	12.97 ± 3.36
6	Henan Mengzhou Golden Corn Co., Ltd., China	dark brown, thick (+++)	83.14 ± 0.99	42.98 ± 0.55	0.16 ± 0.02	8.9 ± 0.6	14.66 ± 0.05

[a] Determination by micro-Kjeldahl method. [b] Measured on the basis of NaOH titration.

compound discovery. Therefore, GC-TOFMS and multielemental fingerprint coupled with chemometrics can provide integrative quality evaluation of CSL samples used in the 2-KLG fermentation.

The aim of this study was to comprehensively investigate variations of different CSLs and explore the relationship between compositions of CSL and 2-KLG production. The compositions of CSL were determined by gas chromatography with time-of-flight mass spectrometry (GC-TOFMS) and ICP-AES. A comparative 2-KLG fermentation using different CSL samples from six suppliers and OPLS-DA enabled the discovery of potential marker compounds relating to 2-KLG production. In addition, this study demonstrated a unique platform for further exploration of the relationship between CSL quality and 2-KLG production.

MATERIALS AND METHODS

Samples. CSL samples from six suppliers in China were collected in this study; further details are given in Table 1. A stock sample of CSL from each origin was maintained at −20 °C until analysis.

Chemicals. Ultrapure water from a Milli-Q system (Millipore, Bedford, MA) was used throughout this experiment. Pyridine (98%), methoxamine hydrochloride (98%), and *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA, 98%) were purchased from Sigma-Aldrich (St. Louis, MO). Succinic acid-d_4 (98%, Sigma-Aldrich) served as internal standard (IS). Chromatographic grade methanol was obtained from the Kang Kede Chemical Corp. (Tianjin, China). Standard solutions of 10 elements including K, P, Mg, Na, Ca, Fe, Zn, Mn, Cr, and Cu were prepared by dilutions of 1000 mg/L stock solutions (Tianjin Chemical Corp., China) prior to use. All acids such as HNO_3 and $HClO_4$ were of analytical reagent grade (Tianjin Chemical Corp.). All glassware and equipment were soaked with 10% HNO_3 at least overnight and then were rinsed with ultrapure water prior to use.

Strains and Medium. *K. vulgare* and *B. megaterium* from Biology Science and Engineering College, Hebei University of Science and Technology, were used in the experiment. Seed culture medium consists of 20 g/L L-sorbose, 3 g/L CSL, 10 g/L peptone, 3 g/L yeast extract, 3 g/L beef extract, 1 g/L urea, 1 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, and 1 g/L $CaCO_3$ (pH 6.8), whereas the solid medium had another 20 g/L of agar. Sorbose−CSL fermentation medium was made of 80 g/L L-sorbose, 20 g/L CSL, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 12 g/L urea, and 5 g/L $CaCO_3$ (pH 7.0).

Inoculum and Analysis for 2-KLG. The agar plat cultures of *K. vulgare* and *B. megaterium* were inoculated into 50 mL of seed culture medium at 200 rpm and 30 °C for 48 and 24 h, respectively. The seeds were mixed at 8:3 (*K. vulgare*/*B. megaterium*, v/v),[16] and then the mixture was transferred into the sorbose−CSL fermentation medium. Three duplicates were prepared in this step. Fermentations were carried out in a 200 mL flask containing 50 mL of medium for 72 h at 200 rpm and 30 °C. 2-KLG contents in the culture broth were periodically measured at 0, 24, 48, and 72 h after the addition of the medium by HPLC. Comparative studies were performed under the same conditions except CSL.

2-KLG was quantified by HPLC (Waters Corp., Milford, MA) with a refractive index (RI) detector. Samples were diluted 1:10 with the solvent mobile phase (5 mM sulfuric acid), followed by filtration through a 0.22 μm porous membrane.[17] An Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad, CA) was used for separation at the column temperature of 65 °C using 5 mM H_2SO_4 as eluent. Samples were eluted at a flow rate of 0.6 mL/min. The retention time was approximately 10 min.

Sample Preparation and GC-TOFMS Analysis. We investigated the components soluble in water. The CSL sample (1 g) was first centrifuged at 10000 rpm for 5 min, followed by filtration through a 0.22 μm porous membrane. The filtrate was prepared by 1:20 dilution using Milli-Q water. Then the diluted sample (20 μL) was added with 30 μL of IS (0.040 mg/mL in methanol) before evaporation to dryness in a vacuum chamber to correct minor variation during analysis.[18] Two-stage chemical derivatization was performed.[19] First, samples were dissolved in 50 μL of methoxamine hydrochloride (20 mg/mL in pyridine) and incubated at 30 °C for 90 min. Then, 80 μL of MSTFA was added, and the sample was incubated at 37 °C for 30 min for trimethylsilylation. Four replicates were performed for each sample.

GC-TOFMS Analysis. GC-TOFMS analysis was performed subsequently. One microliter of derivatized sample was injected by Agilent 7683 autosampler into the GC (Agilent 6890). Separation was achieved on a fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm DB-5MS stationary phase, J&W Scientific, Folsom, CA) at a split ratio of 20:1. Helium was used as carrier at a constant flow rate of 0.6 mL/min. The injector temperature was set at 280 °C. The oven temperature was programmed as follows: 70 °C for 2 min, increased to 290 °C at 5 °C/min ramp, and finally held for 10 min at 290 °C. The column effluent was introduced into the ion source (250 °C) of TOFMS (Waters Corp.). For MS detection, electron ionization mode at 70 eV was used. The mass range was from m/z 50 to 800 with a scan time of 0.5 s.

Data Preprocessing. Essential compounds were qualitatively and quantitatively determined by Masslynx software (version 4.1, Waters Corp.).[20] Automatic peak detection and deconvolution were performed using a peak width of 2.0 s. The structure of compounds was identified by retention times and mass fragmentation patterns with NIST mass spectral library 2005. Peaks with signal/noise values lower than 10 were rejected. All of the areas of acquired peaks were normalized against IS and DW of CSL samples for further data processing.

Mineralization of the Samples. Each sample was dried at 75 °C in an electric oven to remove moisture and thereafter was transferred to a crucible for dry-ashing in a muffle furnace. The temperature was set at 105 °C at first, then increased to 180 °C, holding for a while, and finally to 450−500 °C until a constant weight was reached. Five grams of ash

was digested in a 4:1 mixture of nitric acid and perchloric acid.[21] Then, 2 mL of HCl was added, and the solution was made up to 20 mL for determining the elements Cr and Cu. For other elements, samples can be directly digested at 1:1 (CSL/mixed acid, w/v) and prepared by 1:100 dilution using Milli-Q water to determine. Four replicates were performed for each sample.

Analysis of the Elements. Ten essential elements were measured by ICP-AES (Varian Inc., VISTA-MPX). To obtain appropriate ICP-AES responses, calibration was carried out by using different concentration level standard solutions as follows: 0.5 mg/L for Zn, Mn, Cr, and Cu; 1 mg/L for Fe; 5 mg/L for Ca; 10 mg/L for Mg and Na; 100 mg/L for K and P. The optical path was continuously purged with nitrogen. The wavelengths used and operating parameters are listed in Table 3. Data acquisition and processing were performed by ICP Expert. software (Varian Inc.). Scanning was repeated three times, and all of the concentrations in the paper are expressed on a dry weight basis.

Multivariate Statistical Analysis. Student's *t* test was used for statistical analysis. The values were considered to be statistically different when the *P* value was <0.05. Furthermore, the data sets derived from GC-TOFMS and ICP-AES were mean-centered and pareto-scaled before OPLS-DA analysis using SIMCA-P 11.5 Demo software (Umetrics, Umeå, Sweden). OPLS-DA can judge the similarities and differences between samples. It was applied to identify the corresponding marker compounds relating to fermentation parameters.[22]

RESULTS

Evaluation CSL Quality in 2-KLG Fermentation. In our research, the quality of CSL was evaluated in 2-KLG fermentation by *K. vulgare* and *B. megaterium*. Under the same conditions except CSL, 2-KLG productions showed differences. After 72 h of coculture, the productions were higher using CSLs 4, 5, and 6 than those using CSLs 1, 2, and 3. Moreover, CSL 4 was 20.9% higher than CSL 3 (data not shown). Student's *t* test was used to investigate the statistical differences in 2-KLG production at 72 h using different CSLs. The production using CSL 4 was the highest, which was considered to be statistically different from other samples ($P < 0.05$); CSLs 1 and 2 were not significantly different ($P > 0.05$); CSLs 5 and 6 were regarded as a group ($P > 0.05$); the production using CSL 3 was the lowest, which was statistically different from others ($P < 0.05$). From the perspective of 2-KLG production, it was believed that CSLs 4, 5, and 6 were better than other raw materials.

Compositions of the CSL. The typical total ion chromatogram (TIC) acquired from the GC-TOFMS analysis of the CSL sample is shown in Figure 2. We can see that there are significant differences among six samples in retention time from 27 to 33 min (Supporting Information, Figure S1). Analyte identification was determined through matching to library standards with match values provided to indicate the confidence in the identification. Carbohydrates were identified with reference to analysis of sugars by GC-MS[23] apart from *m*/*z*. Fifty-six compounds (matching score above 800) were further relatively quantified and are listed in Table 2. Under the chromatographic conditions described above, six groups of compositions were separated, including 18 kinds of amino acids, 13 kinds of organic acids, 8 kinds of nitrogen heterocycles, 7 kinds of carbohydrates, 6 kinds of alcohols, and 4 kinds of amines. Analysis of the distributions of constituents showed that amino acid contents were the highest. This was in accordance with the study carried out by Christianson et al.[6] Glucose and fructose are the predominant mono saccharides. When six CSL samples were compared, the contents in CSL 6 were higher than others, which may be caused by the high DW level. Glucose and fructose in CSL 3 were the highest.

The 10 elements listed in Table 3 were determined by ICP-AES. Appropriate wavelengths were selected to ensure accurate detection of multiple elements. As shown in Table 3, the elements were found at various concentrations and ranged from 2 mg/kg to 60 g/kg. The results showed that the CSL had a high level of K and P, followed by Mg, Na, Ca, and Fe and a low level of Cr, Mn, Zn, and Cu. Student's *t* test was used to investigate the statistical differences of elements among CSL samples. It was observed that K, Mg, and P contents in CSL 5 were significantly lower than others, and Na contents in CSLs 2 and 3 were higher. Also, some metal elements such as Fe, Mn, Zn, Cu, and Cr were also determined, which may play a physiological role in the fermentation process. The contents of Fe in CSL 6, Zn in CSL 5, Mn in CSLs 2 and 5, Cr in CSLs 3 and 6, and Cu in CSLs 4 and 5 were considered to be statistically different from other samples ($P < 0.05$). The different contents of various compositions suggested that they might have different fermentation behaviors.

Multivariate Statistical Analysis Using OPLS-DA. OPLS-DA was used to develop a model to discriminate CSLs and discover marker compounds. In the study, a five-component OPLS-DA model ($R^2X = 0.999$, $R^2Y = 0.931$, $Q^2Y = 0.821$) in a supervised mode was generated to suggest the relationship between the *X* variables and *Y* variables. The $\boldsymbol{X}$ matrix consists of the composition data of the six CSL samples in Tables 2 and 3. The $\boldsymbol{Y}$ matrix contains the fermentation parameter, namely, 2-KLG production in Figure 1. The first two PCs explain 81.8% of the total variance. The score plot in Figure 3a shows the similarities and differences of samples. Samples of CSLs 1 and 2 gathered together, whereas CSLs 5 and 6 gathered together. CSL 3 and CSL 4 were alone in the plot. Furthermore, the plot of the first variables of both the components of CSL (t_1) and 2-KLG production (u_1) are shown in Figure 3b, and the high-production and low-production raw materials could be discriminated satisfactorily. The results were accordance with the fermentations at 72 h.

The loading plot (Figure 4a) summarized the influence and correlation structure between variables in both the $\boldsymbol{X}$ matrix and the $\boldsymbol{Y}$ matrix. The further a data point was from the origin, the greater its contribution to $\boldsymbol{Y}$.[18] Furthermore, an S-plot (Figure 4b) combined the contribution/covariance and reliability/correlation from OPLS-DA model and helped to identify marker compounds responsible for the discrimination.[24] For example, the S-plot showed thymine had a high correlation, but the w*c-plot indicated that it was close to the origin. Therefore, thymine was not selected as a marker compound. It was found that the distinguishing components among clusters mainly were some amino acids and reducing sugars. In addition, variable importance in the projection (VIP) of the first component ranked the contribution of each variable to 2-KLG production. VIP indicated the relative influence of each component to 2-KLG production. Components with higher VIP values were more influential. Components were selected as differential signals according to variables with VIP >1 (Figure 4c).[25] Accordingly, 17 compounds, lactic acid, phosphate, glycerol, alanine, proline, 5-oxoproline, valine, glucose, citric acid, glycine, fructose, γ-aminobutyric acid, threonine, isoleucine, tyrosine, aspartate, and serine, may be the critical compounds of CSL used in the 2-KLG fermentation by *K. vulgare* and *B. megaterium*. As the basic nutritional components of microorganisms, amino acids and sugars play many significant roles in metabolism. Therefore,

Table 2. Components Identified in CSL Samples from Different Manufacturers by GC-TOFMS

ID	t_R[a] (min; α-, β-)	compound	m/z[b]	ID	t_R[a](min; α-, β-)	compound	m/z[b]
1	7.20	lactic acid	147.1	29	23.31	ribose	307.17
2	7.62	glycolic acid	147.07	30	23.45	asparagine	231.24
3	8.34	alanine	116.15	31	24.29	ribitol	307.15
4	9.38	oxalic acid	147	32	24.62	xylitol	307.15
5	11.50	valine	144.2	33	24.95	1,4-butanediamine	174.22
6	12.38	urea	189.1	34	25.60	2-ketogluconic acid	292.15
7	13.09	phosphate	299.15	35	26.50	hypoxanthine	265.1
8	13.21	glycerol	205.1	36	26.78	ornithine	142.1
9	13.70	isoleucine	158.2	37	26.85	citric acid	273
10	13.77	proline	142.2	38	27.17	1,5-pentanediamine	174.22
11	14.02	glycine	174.22	39	27.80	adenine	264.1
IS[c]	14.34	succinate	247.1	40	27.97, 28.18	fructose	307.15
12	14.76	glyceric acid	292.15	41	28.26, 28.87	galactose	319.18
13	14.91	uracil	241.1	42	28.35, 28.49	glucose	319.18
14	15.37	maleic acid	245.1	43	29.05	lysine	317.24
15	15.59	serine	204.2	44	29.37	tyrosine	218.15
16	16.27	threonine	218.2	45	30.24	altrose	204.1
17	16.62	thymine	255.11	46	30.77, 30.87	gluconic acid	333.15
18	17.37	β-alanine	116.15	47	30.96	xanthine	353.14
19	19.03	malic acid	233.1	48	31.22	talose	204.1
20	19.28	4-hydroxyproline	230.1	49	31.63	palmitic acid	313.27
21	19.52	erythritol	217.12	50	32.36	inositol	318.16
22	19.72	5-oxoproline	179	51	33.08	guanine	352.15
23	19.83	aspartate	232.23	52	33.17, 33.28	sedoheptulose	319.15
24	20.02	4-aminobutyric acid	174.1	53	34.63	glucitol	319.17
25	20.66	cysteine	220.1	54	35.37	octadecanoic acid	341.29
26	21.31	2-aminocaprylic acid	186.1	55	38.81	uridine	245.15
27	22.32	glutamate	246.24	56	43.95	guanosine	643.3
28	22.36	phenylalanine	218.2				

[a] Retention time. [b] Masses shown are those of the ions selected for tentative identification of individual derivatized components. The ions with the highest intensity are listed. [c] Internal standard.

Table 3. Wavelengths and Concentrations of Different Batches of CSL for the ICP-AES Determination (All Data Converted to Dry Weight by Calculation and Reported as the Mean ± Standard Deviation)[a]

		sample (mg/kg)					
	λ (nm)	CSL 1	CSL 2	CSL 3	CSL 4	CSL 5	CSL 6
K	766.491	55390 ± 1980	60100 ± 2480	60120 ± 1570	61400 ± 1850	33840 ± 760	46080 ± 950
P	213.618	31380 ± 960	31020 ± 1800	28960 ± 990	35240 ± 2610	17660 ± 140	23550 ± 770
Mg	279.553	13950 ± 460	13200 ± 1480	14390 ± 360	16910 ± 750	3100 ± 100	13470 ± 260
Na	589.592	1620 ± 190	22630 ± 190	10770 ± 160	3010 ± 60	1710 ± 110	4130 ± 130
Ca	396.847	1660 ± 70	2330 ± 70	2040 ± 160	1570 ± 120	470 ± 10	1890 ± 230
Fe	238.204	360 ± 20	440 ± 20	350 ± 10	440 ± 10	340 ± 10	230 ± 10
Zn	213.857	180 ± 10	190 ± 17	210 ± 5	220 ± 6	50 ± 1	190 ± 2
Mn	257.61	57 ± 3	81 ± 1	54 ± 1	60 ± 2	29 ± 1	51 ± 1
Cr	267.716	2.4 ± 0.1	2.9 ± 0.5	7.1 ± 0.2	2.6 ± 0.2	2.3 ± 0.4	6.7 ± 0.2
Cu	327.395	6.7 ± 0.1	5.6 ± 0.2	6.0 ± 0.1	1.2 ± 0.4	3.5 ± 0.2	6.5 ± 0.2

[a] Conditions sets: Pwr (KW), 1.00; PlasFlow (L/min), 1.50; AuxFlow (L/min), 1.5; NebFlow (L/min), 0.90. Correlation coefficient limit = 0.995.

they were characteristic compounds of CSL. We compared their contents in six samples. As shown in Figure 5, the concentration of most amino acids in CSL 6 was significantly higher than that in other samples, whereas for glucose and fructose, the concentrations in CSL 3 are the highest in samples.

Identification of Potential Marker Compounds. On the basis of the above results, the effects of potential marker compounds on 2-KLG production were used to guide further investigations. For 17 selected marker compounds from 66 components of CSL (Figure 5), we added single marker

compounds to sorbose−CSL medium, respectively. The results showed that supplementation of culture medium with single markers had a positive effect on 2-KLG production at 24 h, but these markers had no significant effect on 2-KLG production at 72 h (data not shown). We then analyzed the correlation between these markers, and the correlation coefficients between nine potential amino acids were higher ($r > 0.9$). According to our results and the previous report by Zhang et al.,[4] the combination of potential marker compounds was tested as a supplement to sorbose−CSL medium, namely, 0.08 g/L alanine, 0.28 g/L proline, 0.04 g/L valine, 0.36 g/L glycine, 0.18 g/L threonine, 0.05 g/L isoleucine, 0.03 g/L tyrosine, 0.10 g/L aspartate, and 0.28 g/L serine. Compared with the control

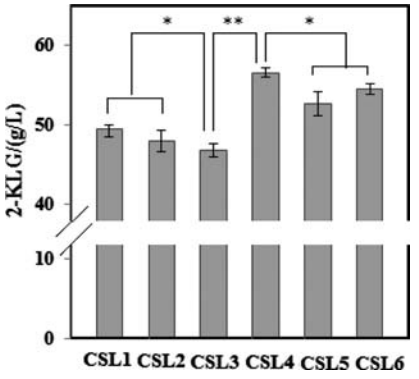

Figure 1. Comparison of 2-KLG productions using different CSLs in 2-KLG fermentation by *K. vulgare* and *B. megaterium* at 72 h. Fermentations were performed under the same conditions except CSL samples. Initial L-sorbose concentration was 80 g/L. Error bars represent standard deviations ($n \geq 3$). Statistically significant differences were determined by Student's *t* test and are indicated with an asterisk (∗, $P < 0.05$; ∗∗, $P < 0.001$).

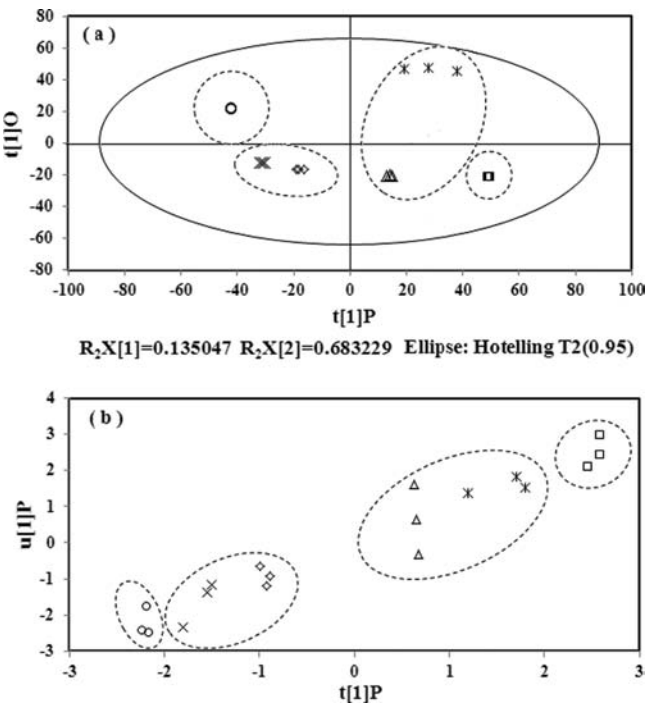

Figure 3. Multivariate statistical analysis of compositions for six CSL samples using OPLS-DA: (a) score plot (t[Comp.1]/t[Comp.2]) of CSL samples; (b) t[Comp.1]/u[Comp.1] plot of experimental CSL samples; (◇) sample 1; (×) sample 2; (○) sample 3; (□) sample 4; (△) sample 5; (∗) sample 6.

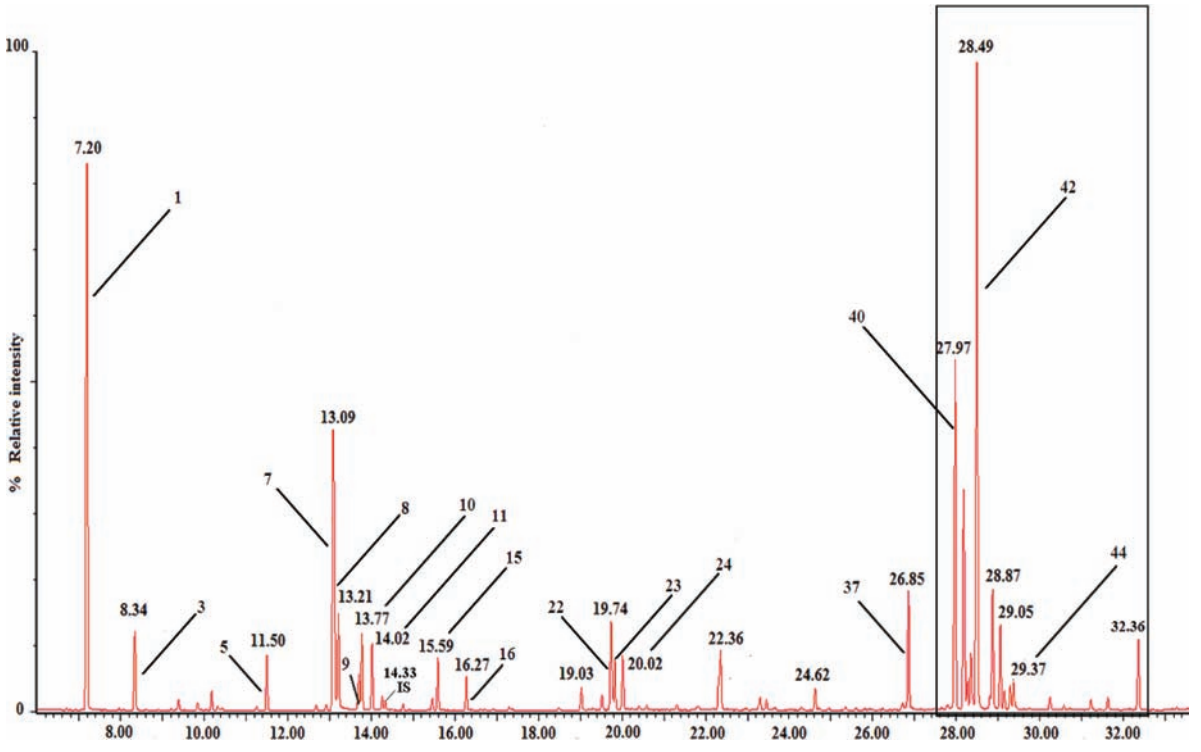

Figure 2. GC-TOFMS total ion current (TIC) chromatogram of CSL. Labeled peaks are corresponding compounds that were considered as potential marker compounds, and the numbers coincide with the ones in Table 2. Significant differences among six CSL samples are marked with boxes. IS, succinic acid-d_4, internal standard used for quantification.

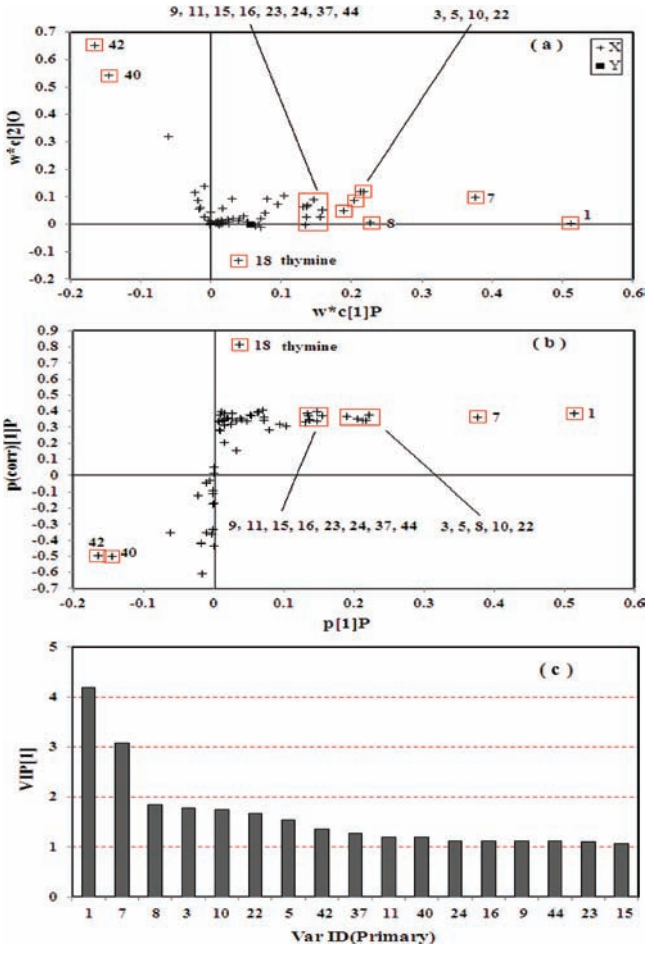

Figure 4. w*c (1)/w*c (2) scatter plot (a) and S-plot (b) from OPLS-DA model of GC-TOFMS and ICP-AES data (crosses) deriving from six CSL samples and 2-KLG production (squares); marker compound candidates are highlighted by squares. (c) Variable importance in the projection of Comp.1 (VIP >1) ranks the contribution of each variable to the 2-KLG production.

sorbose−CSL medium (without any treatment), a higher 2-KLG production at 72 h was achieved by adding the group markers into the medium at zero time (Figure 6). This observation suggested that these compounds were the most influential in 2-KLG production and candidate markers for the future improvement of 2-KLG production.

DISCUSSION

CSL is widely used as a raw material in the pharmaceutical industry, providing organic nitrogen sources for various metabolites. In our research, it played an essential role in fermentation behaviors. We investigated and compared 2-KLG production under identical conditions except CSL in the fermentation by *K. vulgare* and *B. megaterium*. The differences of the production were probably not obvious during the first 24 h. After the formation of spores of *B. megaterium*, 2-KLG production began to differentiate. Because *B. megaterium* secreted some metabolites to the culture broth, the growth of *K. vulgare* was stimulated to enhance 2-KLG production.[26,27] After 72 h of coculture, 2-KLG production was significantly different using the six CSL samples. These facts strongly support our belief that a simple change in the batches or suppliers of CSL could be detrimental in 2-KLG production.

From Table 1, it was noted that the conventional test results of CSL exhibited an agreement with the quality standards, although there were significant differences in DW level. Therefore, these parameters were not enough to ensure CSL stability, and exploring the marker compounds for quality control of CSL would be required. Applying PAT to solving quality control of raw materials problems has already been posed by the U.S. Food and Drug Administration (FDA).[28] We determined 66 compositions in CSL by GC-TOFMS and ICP-AES. Although the analytical approaches are not novel, our research is the first attempt to apply a comprehensive approach to explore the relationship between CSL quality and 2-KLG production and offers information for CSL quality evaluation used in 2-KLG fermentation.

A discussion on the presence or absence of compositions in CSL samples is linked to many factors such as the chromatographic conditions, but the most attractive and powerful aspect of PAT was its ability to identify and detect as many substances as possible simultaneously. In our study, OPLS-DA was carried out to generate information from these high-throughput data. The model enables visualization of the compositions that contribute the most to the discrimination of the six types of CSLs according to 2-KLG production. Six CSL samples can be divided into four groups satisfactorily according to 2-KLG production; that is, different quality raw materials can be discriminated by the proposed components. The loading plots and S-plot from OPLS-DA offered us information about characteristic compounds. Also, the variables that influenced the model construction were studied. The VIP values were computed from the influence on **Y** of every term (**X**'s). Components found to be significantly different among samples were marked (Figure 4) and compared (Figure 5). The marker compounds mainly were some amino acids and reducing sugars. As illustrated in Figure 5, the contents of amino acid in CSL 6 were the highest, followed by CSLs 2, 3, 5, 4, and 1. The contents of glucose and fructose in CSL 3 were highest, followed by CSLs 6, 4, 2, 5, and 1. The significance of variables was found in accordance with those peaks showing an obvious difference in intensity among CSL samples (Figure 2).

Lactic acid and phosphate are known products during steeping. Therefore, the variations in lactic acid and phosphate levels in CSL were caused by intrinsically different levels in the manufacturing process. The high lactic acid is beneficial to proteolysis during steeping.[29] Phosphate is in part a product of dephosphorylation of *myo*-inositol phosphates found in corn steep water.[30] They were donors of ATP providing energy for microorganisms. Citric acid can directly take part in TCA cycle for use in oxidative phosphorylation and other metabolic processes. The pathway also supplied some amino acid and fatty acid precursors. Amino acids and reducing sugars were considered to be nutritional compounds. Alanine and proline were related to the major amino acids of corn proteins,[6] and therefore the differences may be caused by different origins of corn. Alanine and isoleucine may be used for cell growth and enzyme system synthesis. Valine and tyrosine may be used for synthesizing proteins or taking part in amino acid metabolism pathways. Aspartate may provide nutrition, particularly for *B. megaterium*.[31] The presence of threonine in CSL can promote mixed cells growth, and serine is the key component that affects 2-KLG production, which are coincident with the previous discovery.[4]

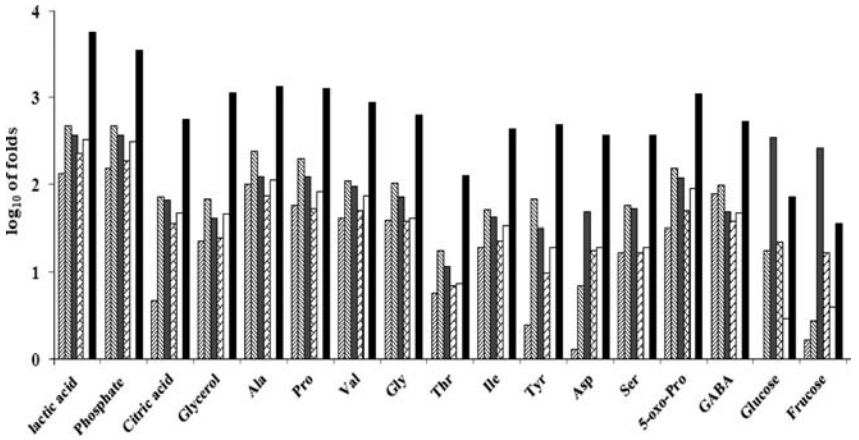

Figure 5. Folds of marker compounds in CSL samples: (forward slashed (/) bars) sample 1; (back-slashed (\) bars) sample 2; (gray bars) sample 3; (brick pattern) sample 4; (white bars) sample 5; (black bars) sample 6. The folds were calculated by normalization of peak area of each compound to internal standard and dry weight of CSL.

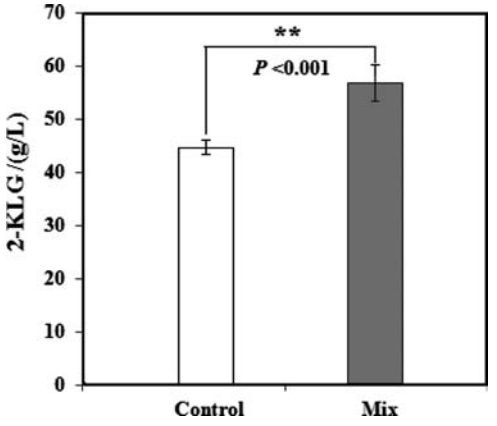

Figure 6. Validation of potential marker compounds by adding combined amino acids to sorbose−CSL medium. The control and mix represent the medium supplied without and with markers, respectively. The mix was made of nine amino acids (0.08 g/L alanine, 0.28 g/L proline, 0.04 g/L valine, 0.36 g/L glycine, 0.18 g/L threonine, 0.05 g/L isoleucine, 0.03 g/L tyrosine, 0.10 g/L aspartate, and 0.28 g/L serine). 2-KLG production at 72 h compared to the control. Each value represents the mean ± standard deviation ($n \geq 3$). **, significantly different from control, $P < 0.001$ (Student's t test).

5-Oxoproline can be converted to glutathione via glutamate. A number of studies have suggested that the reaction of L-sorbose conversion to 2-KLG was catalyzed by sorbose dehydrogenase (SDH) and sorbosone dehydrogenases (SNDH). The sulfhydryl of glutathione can be combined with the dehydrogenase; thus, the two-step dehydrogenation reactions were protected so as to promote 2-KLG accumulation.[32] γ-Aminobutyric acid is present in corn grain in low levels and is considered to be an enzymatic degradation product of glutamic acid during steeping.[33] It was involved in a marker bypassing step of the TCA cycle, which can be converted to succinate or alanine.[34] Glucose and fructose can provide carbon sources for cell growth, but too many reducing sugars may inhibit the accumulation of 2-KLG.[35]

Glycine, glycerol, and proline were considered to be latent marker compounds related to osmotic stress. In the fermentation process, the environmental stress was of primary importance due to the effect on cell membrane compositions and functions. Yang et al.[36] discovered that glycine could affect cell membrane permeability. A higher concentration of glycine is expected by *B. megaterium* to secrete intracellular metabolites, whereas for *K. vulgare*, membrane permeability plays an important role in the secretion of 2-KLG. It is reported that osmotic stress continuously increased with the accumulation of 2-KLG. The mechanism is that glycerol may decrease the destruction of *K. vulgare* at a high 2-KLG concentration.[37] Also, proline is an osmotic protection agent related to 2-KLG production, which is consistent with previous findings.[4]

Additionally, we used a unique approach to screen potential marker compounds from the 17 OPLS-generated species (Figure 4). We added either single or combined markers to the CSL medium at zero time. It was interesting to note that supplementing the sorbose−CSL medium with the single marker compounds had no significant effect on 2-KLG production at 72 h, whereas for the production at 24 h, it had a positive effect. From this there may arise a question: why? This may be caused by the formation of spores of *B. megaterium* after 24 h. It is possible that the influence of *B. megaterium* by secreting some metabolites to the culture broth far exceeded the addition of single markers. Moreover, the inappropriate concentrations of supplemental single markers may inhibit some enzyme activity in the 2-KLG metabolic pathway, destroying the balance of the two-strain symbiotic system.[2,35] Therefore, we require and should add the appropriate concentrations of single markers at appropriate time points to enhance 2-KLG production at 72 h, but this needs further investigation. Indeed, in some situations, it is more complex and various compositions influence and interact with existing raw materials. When combined with nine amino acids, a higher 2-KLG production at 72 h was achieved compared to control (Figure 6). Hence, we can speculate that the nine kinds of amino acids were group marker compounds relating to 2-KLG production used in the 2-KLG fermentation. Further research will focus on the optimization of the potential marker compounds and further verify the correlation between the potential markers identified in the present research and 2-KLG production.

This research marks the first comprehensive investigation on the relationship between the composition of CSL and 2-KLG production by PAT. Seventeen underlying marker compounds shed light on developing a new approach of improving 2-KLG production and provide useful information on CSL quality evaluation used in 2-KLG fermentation. Furthermore, 2-KLG production was the most important processing parameter, which

should be paid more attention in the fermentation process for getting some feedback of raw material quality. The results have revealed that the proposed comprehensive approach can provide an important platform to explore CSL marker compounds relating to 2-KLG production for quality control. This might be a supplemental method for predicting raw material processability behavior.

■ ASSOCIATED CONTENT

Supporting Information. GC-TOFMS total ion current (TICs) chromatograms of six CSL samples obtained on DB-5MS stationary phase. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

PAT, process analytical technology; CSL, corn steep liquor; GC-TOFMS, gas chromatography with time-of-flight mass spectrometry; ICP-AES, inductively coupled plasma-atomic emission spectroscopy; 2-KLG, 2-keto-L-gulonic acid; HPLC, high-performance liquid chromatography; AAS, atomic absorption spectrometry; DW, dry weight; IS, internal standard; MSTFA, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide; EMEA, European Medicines Agency; PCA, principal component analysis; HCA, hierarchical clustering analysis; OPLS-DA, orthogonal partial least-squares discriminant analysis; FDA, U.S. Food and Drug Administration; VIP, variable importance in the projection; SDH, sorbose dehydrogenase; SNDH, sorbosone dehydrogenases.

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