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Development of chemically defined media supporting high cell density growth of *Ketogulonicigenium vulgare* and *Bacillus megaterium*

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ABSTRACT

The immediate precursor of L-ascorbic acid, or vitamin C, is 2-keto-L-gulonic acid (2-KLG). This is commonly produced commercially by *Ketogulonicigenium vulgare* and *Bacillus megaterium*, using corn steep liquor powder (CSLP) as an organic nitrogen source. In this study, the effects of the individual CSLP components (amino acids, vitamins, and metal elements) on 2-KLG production were evaluated, with the aim of developing a complete, chemically defined medium for 2-KLG production. Forty components of CSLP were analyzed, and key components were correlated to biomass, 2-KLG productivity, and consumption rate of L-sorbose. Glycine had the greatest effect, followed by serine, biotin, proline, nicotinic acid, and threonine. The combination of 0.28 g L^{-1} serine, 0.36 g L^{-1}glycine, 0.18 g L^{-1} threonine, 0.28 g L^{-1}proline, 0.19 g L^{-1}nicotinic acid, and 0.62 mg L^{-1}biotin in a chemically defined medium produced the highest maximum biomass concentration (4.2×10^9 cfu mL^{-1}), 2-KLG concentration (58 g L^{-1}), and yield (0.76 g g^{-1}) after culturing for 28 h.

1. Introduction

With an estimated global production of 110,000 tons per year, the market for L-ascorbic acid, commonly known as vitamin C, is becoming increasingly more competitive (Macauley et al., 2001). The well-known two-step production process, originally developed in China in the 1960's (Yin et al., 1997), was the first unique process to compete with the previously employed Reichstein process and is currently used by all Chinese manufacturers. In the two-step fermentation process, *Ketogulonicigenium vulgare* (previously identified as *Gluconobacter oxydans*) (Yang et al., 2006) and *Bacillus megaterium* are used to synthesize the L-ascorbic acid precursor 2-keto-L-gulonic acid (2-KLG). A high 2-KLG concentration (75.8 g L^{-1}) and yield (94.8%) can be achieved by providing L-sorbose to *K. vulgare* and *B. megaterium* during 72 h of cultivation (Xu et al., 2004), with the 2-KLG yield significantly increased by the use of two bacterial strains, rather than just *K. vulgare* (Feng et al., 2000). *B. megaterium* is generally thought to act as a companion strain that synthesizes and secretes metabolites into the fermentation broth, stimulating the growth of *K. vulgare* and increasing the 2-KLG production (Zhao et al., 2008). Recently, genome sequences for *K. vulgare* and *B. megaterium* have enabled the development of metabolic models and opened up novel "omics" strategies for examining cellular metabolism during fermentation. All of these strategies are greatly facilitated by the use of a chemically-defined medium, in which metabolism is more easily defined.

As it lacks various biosynthetic pathways, *K. vulgare* generally requires a medium rich in nutrients. A nutrient-rich environment can be provided by a semi-defined medium (SDM), formulated mostly with defined chemicals (except for corn steep liquor powder (CSLP)) (Takagi et al., 2009). However, using an SDM in physiological studies focusing on metabolism and regulation makes data more difficult to interpret as consumption of the numerous intermediary metabolites produced during biosyntheses of macromolecules is not easily quantified. For this reason, a chemically defined medium (CDM) that supports reasonable cell growth can be of great help in the study of gene regulation and metabolic fluxes. By systematically adding or removing components from the CDM formulation, the specific nutritional and regulatory requirements for growth and targeted metabolic pathways can be determined. Uncertainties due to the complicated interactions among complex components can be minimized or at least more easily understood, and the culture environment is more reproducible.

In this study, 2-KLG fermentation by *K. vulgare* and *B. megaterium* is used as a model for investigating the qualitative

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physiological role of CSLP composition on metabolic behaviors under conditions of industrial fermentation. In addition, a CDM was developed that supports growth of *K. vulgare* and *B. megaterium* that is comparable to or exceeds growth on a semi-defined medium.

2. Methods

2.1. Bacterial strains and media

The strains of *K. vulgare* and *B. megaterium* used in this study were obtained from Jiangsu Jiangshan Pharmaceutical Co. Ltd., and stored at the Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University.

Medium A for seed cultures contained (g L^{-1}): L-sorbose 20, yeast extract 3, peptone 10, beef extract 3, CSLP 1.5, urea 1, $CaCO_3$ 1, $MgSO_4$ $7H_2O$ 0.2, KH_2PO_4 1. For plate cultures, 2% agar was added. Medium B for fermentation contained (g L^{-1}): L-sorbose 80, urea 12, CSLP 5, $MgSO_4$ $7H_2O$ 0.1, KH_2PO_4 1. Synthetic medium C contained (g L^{-1}): L-sorbose 80, urea 12, $MgSO_4$ $7H_2O$ 0.1, KH_2PO_4 1, aspartate 0.10, glutamate 0.26, histidine 0.05, arginine 0.05, alanine 0.19, tyrosine 0.03, cysteine 0.01, valine 0.10, methionine 0.04, phenylalanine 0.06, isoleucine 0.06, leucine 0.16, lyscine 0.05; metal ion solutions 1 mL L^{-1}, vitamin solutions 0.1 mL L^{-1}, pH 6.7–7.0. The concentrations of serine, glycine, threonine, proline, nicotinic acid, and biotin were adjusted according to the experimental design.

Metal ion solutions were (g L^{-1}): $ZnCl_2$ 1.75, $FeCl_3$ 4.61, $MnCl_2$ 0.85, $CuCl_2$ 0.06, KCl 442.60, NaCl 44.71, $MgCl_2$ 301.16, $CaCl_2$ 17.39, KH_2PO_4 148.68. Vitamin solutions were (g L^{-1}): vitamin B1 0.04, vitamin B2 0.30, vitamin B6 1.20, vitamin B12 0.06, pantothenic acid 1.15, and folic acid 0.01.

In culture flasks, 5 g L^{-1} $CaCO_3$ were added to buffer the fermentation broth. The initial pH of all media was adjusted to 7.0. L-sorbose and $CaCO_3$ were sterilized separately prior to adding to the medium.

Table 1
Eighteen different samples of CSLP.

No.	Manufacturer	Source
1	Dezhou Fuyuan Biology Starch Co., Ltd.	Pingyuan, Shandong, China
2	Wuhan Galaxy Chemical Co., Ltd	Wuhan, Hubei, China
3	Shandong Yuncheng Health Biotechnology Co., Ltd.	Yuncheng, Shandong, China
4	Henan Julong Starch Industrial Co., Ltd	Ruzhou, Henan, China
5	Binzhou Gangfa Biotechnology Co., Ltd.	Binzhou, Shandong, China
6	Shangdong Fulldail Biotechnology Co., Ltd.	Gaoqing, Shandong, China
7	Wuhan Bioco Sci. & Tech. Dev. Co.,Ltd.	Wuhan, Hubei, China
8	Wuhan Jiabao Sugar Co., Ltd	Wuhan, Hubei, China
9	Shandong Zouping Hebanshan Biotechnology Co., Ltd.	Zouping, Shandong, China
10	Luzhou Bio-Chem Technology Co., Ltd. (Sichuan)	Pengshan, Sichuan, China
11	Luzhou Bio-Chem Technology Co., Ltd. (Henan)	Zhumadian, Henan, China
12	Shanghai Xiwang Starch sugar Co., Ltd.	Shanghai, China
13	Xinyi Henghui Starch sugar Co., Ltd.	Xinyi, Jiangsu, China
14	Shandong Fufeng Fermentation Co.,Ltd	Lunan, Shandong, China
15	Weifang Huike Chemical Co.,Ltd.	Shouguang, Shandong, China
16	Shandong Kaideli Sugar Industry Co., Ltd.	Tancheng, Shandong, China
17	Fucheng Fuyuan Food Co., Ltd.	Fucheng, Hebei, China
18	Jiangxi Jinbiyuan Industrial Co., Ltd.	Nanchang, Jiangxi, China

2.2. Culture conditions

The seed culture inoculated from a slant was cultivated at 30 °C, 200 rpm, in a 750-mL flask containing 75 mL medium A on a reciprocal shaker for 32 h (*K. vulgare*) or 9 h (*B. megaterium*). The seeds were mixed and cultured for another 18 h, and then the mixture of *K. vulgare* and *B. megaterium* was transferred into the fermentation medium (Zhang et al., 2010). Fermentations were carried out in 750-mL flasks containing 75 mL medium B or in a 7-L jar fermentor (KF-7 L, Korea Fermentor Co., Inchon, Korea) with 4 L of medium B. The amount of inoculum was 10% (v/v). Culture flasks were incubated for 68 h at 200 rpm. In fermentor cultures, the pH was automatically controlled to 7.0 with 8 M NaOH solution, with stirring at 400 rpm and air flow of 1.5 L min^{-1}. All experiments were performed in triplicate. All cultivations were at 30 °C (Takagi et al., 2009).

2.3. CSLP sample

The 18 CSLP samples were collected from eight different habitats (Shandong (8), Hubei (3), Henan (2), Shanghai (1), Jiangsu (1), Hebei (1), Sichuan (1), and Jiangxi (1)) in China; further details are given in Table 1.

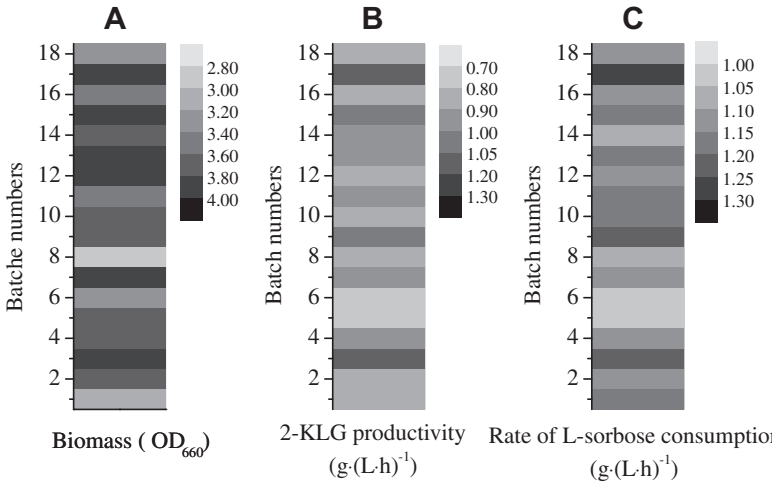

Fig. 1. Fermentation parameters from different CSLP batches. (A) Biomass, (B) 2-KLG productivity, (C) Rate of L-sorbose consumption.