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# Economically enhanced succinic acid fermentation from cassava bagasse hydrolysate using *Corynebacterium glutamicum* immobilized in porous polyurethane filler

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## HIGHLIGHTS

- CBH was used as carbon source for fermentation of succinic acid.
- Mixed alkalis (NaOH and $Mg(OH)_2$) were used as a novel method for regulating pH.
- The *C. glutamicum* strains were first immobilized in porous polyurethane filler.
- Using CBH and mixed alkali in the immobilized system for succinic acid production.

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## ABSTRACT

An immobilized fermentation system, using cassava bagasse hydrolysate (CBH) and mixed alkalis, was developed to achieve economical succinic acid production by *Corynebacterium glutamicum*. The *C. glutamicum* strains were immobilized in porous polyurethane filler (PPF). CBH was used efficiently as a carbon source instead of more expensive glucose. Moreover, as a novel method for regulating pH, the easily decomposing $NaHCO_3$ was replaced by mixed alkalis (NaOH and $Mg(OH)_2$) for succinic acid production by *C. glutamicum*. Using CBH and mixed alkalis in the immobilized batch fermentation system, succinic acid productivity of 0.42 g $L^{-1}$ $h^{-1}$ was obtained from 35 g $L^{-1}$ glucose of CBH, which is similar to that obtained with conventional free-cell fermentation with glucose and $NaHCO_3$. In repeated batch fermentation, an average of 22.5 g $L^{-1}$ succinic acid could be obtained from each batch, which demonstrated the enhanced stability of the immobilized *C. glutamicum* cells.



## 1. Introduction

Recently, succinic acid has gained attention as an important chemical because it can be used for the synthesis of many basic general chemicals. At present, the primary method for succinic acid production is chemical synthesis, using fossil fuels, which is associated with a high environmental cost, particularly higher $CO_2$ emissions. As the price of fossil fuel has skyrocketed and the consciousness of environmental awareness has increased, biological processes for succinic acid production will become more economical and acceptable (Shohei et al., 2008).

Succinic acid is an end product during anaerobic fermentation by some anaerobic and facultative anaerobic microorganisms. Strain is one of the key points of succinic acid biosynthesis; most bacteria and fungi can produce succinic acid (Song and Lee, 2006). At present, research on industrial succinic acid fermentation strains are mainly focused on anaerobic bacteria, such as *Anaerobiospirillum succiniciproducens* (Lee et al., 2003), gram-negative bacteria, such as *Actinobacillus succinogenes* (Guettler et al., 1999), and *Escherichia coli* (Lin et al., 2005; Skorokhodova et al., 2013). Moreover, *Corynebacterium glutamicum* has received much attention in this field in recent decades. Under anaerobic conditions, cell growth of *C. glutamicum* was inhibited, but the cells remained capable of metabolizing carbohydrate to produce organic acid, such as L-lactic acid, succinic acid, and acetic acid. The metabolic pathway used by *C. glutamicum* to produce lactic acid and succinic acid under anaerobic condition is shown in Fig. 1 (Chen et al., 2012). Large amounts of alkaline neutralizer are required

*Abbreviations:* CBH, cassava bagasse hydrolysate; PPF, porous polyurethane filler; PPFS, porous polyurethane fillers; DCW, dry cell weight.

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to maintain the pH during succinic acid fermentation. The majority of studies on succinic acid production by *C. glutamicum* have used $NaHCO_3$ as alkaline neutralizer to achieve high product concentrations (Inui et al., 2004; Li et al., 2010). Nevertheless, $NaHCO_3$ is not stable when temperature is over 50 °C, so use of $NaHCO_3$ as a neutralizer is limited in industrial succinic acid fermentation. There have been no reports regarding the use of mixed alkalis (e.g., NaOH and $Mg(OH)_2$) as alkaline neutralizers for regulating pH for succinic acid production by *C. glutamicum*.

Carbon sources often constitute a large portion of the raw material costs in biological fermentation, and much effort has gone into identifying cheap and renewable sources that can replace such expensive sources. Cassava bagasse, the fibrous residue from the industrial processing of cassava for starch extraction, is generated in large quantities in many countries and is treated as solid waste, because bagasse can be used only as low-value animal feed or must be disposed into landfills. Biological conversion of cassava bagasse has been previously studied for production of organic acids, butanol, and aromatic compound production (Bramorski et al., 1998; Carta et al., 1999; Lu et al., 2012; Thongchul et al., 2009), but has never been reported for succinic acid production by *C. glutamicum*. Utilizing cassava bagasse for succinic acid production could lower the substrate costs and could add value to the cassava processing industry while reducing environmental pollution caused by bagasse disposal.

Immobilization of cells onto inert materials has been an alternative means of high biomass retention. When provided with nutrients, the cells divide within and on the core of the matrix, also releasing some of the progeny in the medium. To date, studies of succinic acid production by *C. glutamicum* relied on centrifugation of the aerobic medium to collect the cell mass (Shohei et al., 2005, 2008). This procedure was time consuming and liable to be contaminated by strains suspended in the air. To solve this problem, some researchers have attempted the use of immobilization techniques with *C. glutamicum* (Amin and Al-Talhi, 2007; Chu et al., 1996; Jun et al., 2007; Vijayaraghavan et al., 2008). Most studies employed entrapment methods using a polyelectrolyte complex gel and polysulfone matrix (Chu et al., 1996; Vijayaraghavan et al., 2008). In gel-entrapping methods, however, the limitation of carbon dioxide supply by diffusional resistance of the polyelectrolyte complex gel and polysulfone matrix may decrease the fermentation rate and/or succinic acid transformation efficiency (Corona-González et al., 2014).

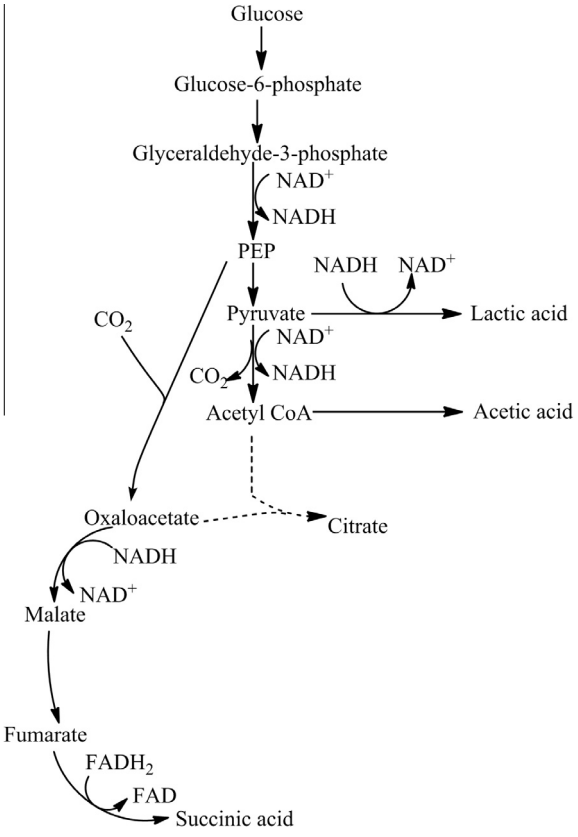


**Fig. 1.** Metabolic pathways of *C. glutamicum* under oxygen-deprivation conditions. L-Lactic acid, succinic acid, and acetic acid are the main products during the anaerobic fermentation.

Hence, in this work, we proposed a natural attachment method by using porous polyurethane filler (PPF) as a carrier for *C. glutamicum* immobilization. PPF has macropores larger than hundreds of microns and a pore volume fraction greater than 0.9. To our knowledge, succinic acid production has not been achieved using *C. glutamicum* with PPF (John et al., 2007; Zhu et al., 1996) to date.

Moreover, in this study, mixed alkalis (NaOH and $Mg(OH)_2$) were used as a novel method for the first time for regulating pH for succinic acid production by *C. glutamicum*. A system using cassava bagasse hydrolysate (CBH) as the carbon source and mixed alkalis as the alkaline neutralizer was developed here to achieve economical succinic acid production by *C. glutamicum* immobilized in PPF.

## 2. Methods

### 2.1. Preparation of CBH

Cassava bagasse, obtained from a cassava-processing factory in Guangdong, China, was dried and mechanically milled to a fine powder (about 50–100 μm in diameter). Before saccharification, 200 g of dried cassava bagasse powder was mixed with 1800 mL water (corresponding to a 10% (w/w) solid loading) and liquefied (using α-amylase 20,000 U $mL^{-1}$ and 15 U $g^{-1}$ dry cassava bagasse) at 86 °C for 2 h in a 5-L conical flask. Then, the liquefied cassava was cooled to 55 °C, after which commercial glucoamylase (100,000 U $mL^{-1}$, from Zhiyi, Shanghai, China) was aseptically added at a 200 U $g^{-1}$ dry cassava bagasse loading, to hydrolyze the cooked starch content into glucose. This enzymatic hydrolysis process was operated at 55 °C, pH 4.5, and 200 rpm for 2 h. Then, cellulase (10,000 U $g^{-1}$, from Zhiyi, Shanghai, China) was added into the mixture at a loading rate of 0.1 mL $g^{-1}$ dry cassava bagasse to hydrolyze the remaining cellulose into glucose. This process was operated at 50 °C, pH 5.0, and 200 rpm for 24 h. 2 M HCl and 4 M NaOH solutions were used for pH adjustments only prior to each enzymatic hydrolysis. No pH adjustment or buffer solution was used during the enzymatic hydrolysis process, as no significant pH change was observed. After hydrolysis, the mixture was centrifuged at 7000×*g* for 15 min to remove the insoluble substances. The clear liquid, CBH, contained 43.5 g $L^{-1}$ glucose, 1.4 g $L^{-1}$ xylose, and trace amounts of arabinose, acetic acid, and lactic acid.

### 2.2. Microorganism and culture medium

A hyper-succinic acid-producing *C. glutamicum* strain, strain 534, derived from ATCC 13032 through mutagenesis and adaptation in PPF, was used in this study. The stock culture of this mutant strain was stored in a 15% glycerol stock solution in a −80 °C freezer. To prepare the seed culture for fermentation studies, the strain was transferred from a Luria broth (LB) culture plate into an Erlenmeyer flask (500 mL) containing 30 mL seed medium, and incubated aerobically at 30 °C for 12–15 h, until the cells were highly active. The seed culture medium, containing a carbon source (20 g $L^{-1}$ glucose),