Bioresource Technology 107 (2012) 363–367

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Production of fumaric acid by simultaneous saccharification and fermentation of starchy materials with 2-deoxyglucose-resistant mutant strains of *Rhizopus oryzae*

Yuefang Deng, Shuang Li, Qing Xu, Min Gao, He Huang *

State Key Laboratory of Material-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering, Nanjing University of Technology, No. 5 Xinmofan Road, Nanjing 210009, PR China

ARTICLE INFO

Article history:
Received 5 September 2011
Received in revised form 26 November 2011
Accepted 26 November 2011
Available online 6 December 2011

Keywords:
Fumaric acid
Rhizopus oryzae
Cornstarch
Degermed corn powder
Simultaneous saccharification fermentation (SSF)

ABSTRACT

A mutant strain with high glucoamylase activity and insensitive to catabolite repression was developed to produce fumaric acid by simultaneous saccharification and fermentation (SSF) of starch without additional commercial glucoamylase supplementation. A series of mutant strains resistant to the non-metabolizable and toxic glucose analog 2-deoxyglucose (2-DG) were obtained by implanting nitrogen ion (N^+) into *Rhizopus oryzae* ME-F12. Among them, the best mutant strain DG-3 produced 39.80 g/L fumaric acid, which is 1.28-fold of that produced by ME-F12, and exhibited higher glucoamylase activity during SSF. Higher fumaric acid production (44.10 g/L) was achieved when the initial total sugar concentration of cornstarch was 100 g/L. During SSF of cheap, raw bioresource-degermed corn powder (100 g/L total sugar) by DG-3, the maximum fumaric acid concentration and productivity were 32.18 g/L and 0.44 g/(L h), respectively.

1. Introduction

Fumaric acid is a four-carbon unsaturated dicarboxylic acid widely used in chemical, food, and pharmaceutical industries. The fumaric acid currently in use is produced through the catalytic oxidation of benzene via fossil resources. Benzene is a well-known carcinogenic substance, so new methods of producing fumaric acid for food and pharmaceutical industries are essential. Fumaric acid obtained via the bioconversion of renewable feedstock has received extensive attention worldwide (Goldberg et al., 2006). Gangl et al. (1990) pointed out that one of the greatest obstacles restraining the industrialization of fumaric acid fermentation is the cost of raw materials. Using purified sugars, such as glucose and sucrose, as feedstocks for fumaric acid production is costly. The utilization of starchy materials instead of expensive refined sugars is more economical. Conventional fermentative production of fumaric acid from starch materials entails the use of acid- or enzyme-hydrolyzed starchy materials, such as potato, cassava, and corn flours via separated starch hydrolysis and fermentation (SHF) operation (Carta et al., 1999; Moresi et al., 1992). Another process called simultaneous saccharification and fermentation (SSF) has been developed and successfully applied in ethanol (Ohgren et al., 2007) and lactic acid (Romani et al., 2008) production. SSF can solve the inhibition of high sugar concentration in the medium and reduce the cost of production. In SSF, hydrolyzing enzymes are added along with the inoculum. Considering that the addition of commercial saccharifying enzyme is required during fermentation, SSF that uses a fumaric acid-producer strain which produces glucoamylase has several advantages in terms of operational cost and space.

Rhizopus oryzae is one of the best-known producers of fumaric acid (Carol et al., 2008). As a filamentous fungus, *R. oryzae* itself can secrete glucoamylase, which has been used in SSF process for lactic acid production (Hang, 1989). However, the yield is always low. If the activity of the glucoamylase of *R. oryzae* could be improved, the SSF process for fumaric acid production from raw starch material without the addition of commercial glucoamylase might be established.

One of the reported causes of low glucoamylase yield is the catabolite repression of enzyme biosynthesis due to glucose or metabolizable compounds. 2-deoxyglucose (2-DG), a glucose analog, inhibits the growth of yeasts and filamentous fungi, which, when incorporated into a suitable medium, do not cause the catabolite repression of mutant strains, allowing only mutated strains resistant to this form of suppression to form colonies (Dillon et al., 2006). The productivity of catabolic enzymes, such as glucoamylase in *Aspergillus terreus* (Ghosh et al., 1991), β-glucosidase in *Aspergillus niger* (Sarangbin et al., 1993), and cellulase in *Trichoderma citrinoviride* (Chandra et al., 2009) is improved by

* Corresponding author. Tel./fax: +86 25 83172094.
E-mail address: biotech@njut.edu.cn (H. Huang).

0960-8524/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2011.11.117

the induction of 2-DG-resistant mutant strains less sensitive to catabolite repression. However, to our knowledge, the foregoing has not been described as a strategy for isolating a mutant of *R. oryzae* capable of producing a high yield of glucoamylase. Therefore, the objective of the present work is to enhance the activity of the glucoamylase of *R. oryzae* and to develop an SSF process for fumaric acid production from starch materials without commercial glucoamylase supplementation.

2. Methods

2.1. Chemicals

Thermostable α-amylase and glucoamylase with activities of 1.0×10^5 U/mL and 1.9×10^5 U/mL, cornstarch, corn powder, and degermed corn powder were provided by COFCO Biochemical (ANHUI) Co. Ltd. (Bengbu, China). Corn powder and degermed corn powder had starch contents of 72–76% (w/w). The 2-DG was purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). All other chemicals were of analytical grade and commercially available.

2.2. Preparation of liquefied starchy material

The starchy materials were gelatinized and liquefied with α-amylase. The starchy materials were suspended and gelatinized in deionized water at 20% (w/v) concentration. The pH of the suspension was adjusted to 6.0 using 2 mol/L HCl. Excess thermal α-amylase (4000–5000 U/g dry substrate bases) was added to the suspension, and the resulting mixture was heated to 95 °C in a water bath. The residual starch was measured by color reaction of iodine. Liquefaction was considered completed when the blue coloration of the iodine test faded. For the corn powder and degermed corn powder, the liquefied materials were filtrated with two layers of gauze. The solution was diluted to the required substrate concentration for fermentation.

2.3. Microorganism and medium

R. oryzae ME-F12, the mutant of *R. oryzae* ATCC 20344, was used for the mutation in the current study (Xu et al., 2010). *R. oryzae* ME-F12 was grown on potato-dextrose agar (PDA) slants at 35 °C for 7 days. The fungal spores in the slants were suspended in sterilized water maintained at 4 °C. The seed medium was composed of 30 g/L glucose, 2 g/L urea, 0.6 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.11 g/L $ZnSO_4$ $7H_2O$, and 0.0088 g/L $FeSO_4{\cdot}7H_2O$ with pH 2.5. The fermentation medium was composed of liquefied cornstarch containing 60 g/L–120 g/L total sugar, 0.1 g/L–0.125 g/L urea, and 40 g/L–60 g/L $CaCO_3$, supplemented with other ingredients similar to those of the seed medium. The fermentation medium using liquefied corn powder or degermed corn powder as carbon resource contained 100 g/L total sugar and 50 g/L $CaCO_3$ without other ingredients.

2.4. Seed culture and SSF process

Seed culture and fumaric acid production (with SSF) fermentation were carried out in a 250 mL flask with 50 mL medium at 200 rpm at 35 °C temperature. The SSF batch cultivations from degermed corn powder was conducted in a 3 L stirred bioreactor (NBS, NEW Brunswick, USA) containing 2 L clarifying solution medium at 35 °C, 1 VVM, and 400 rpm for 84 h. For the seed culture, 1 mL spore suspension (2×10^7/mL) was inoculated into the flask. For simultaneous saccharification and fermentation experiments, 10% (v/v) of seed cultured *R. oryzae* was employed without the addition of commercial glucoamylase.

2.5. Mutagenesis and selection of 2-DG-resistant mutants

The strain *R. oryzae* ME-F12 was mutagenized by nitrogen ion implantation. About 100 μL spore suspension was spread to form a single-cell layer on a sterilized Petri dish and dried in the hood. The dishes were implanted by N^+ beams with a dose of 2×10^{15} ions/cm at 10 keV energy. The operation pressure in the target chamber was about 10^{-3} pa. The implanted samples were diluted by 1 mL sterilized water and plated onto 2-DG/glycerol agar medium (LDG) comprising 30 g/L glycerol, 2 g/L urea, 0.6 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.11 g/L $ZnSO_4$, 0.088 g/L $FeSO_4{\cdot}7H_2O$, and 20 g/L agar with the addition of 2-DG. After cultivation for 2 days, the growing colonies were transferred onto PDA slants. Cultivation was carried out for 7 days to allow the conidiation of each strain. For the selection of stable DG-resistant mutant strains, the conidia were inoculated on 2-DG/glycerol agar medium. The growing colonies were picked up and maintained on PDA slants. The conidia of these strains were inoculated on 2-DG/glucose agar medium (GDG) comprising 30 g/L glucose, 2 g/L urea, 0.6 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.11 g/L $ZnSO_4$, 0.088 g/L $FeSO_4{\cdot}7H_2O$, and 20 g/L agar with the addition of 2-DG, and cultivated for 9 days. Colonies showing growth on LDG and producing conidia on GDG were selected and tested for fumaric acid.

2.6. Assay of glucoamylase

Glucoamylase activity was measured according to the method of Abe et al. (1988) with some modifications. The reaction mixture containing 1.0 mL of 2% (w/v) soluble starch, 0.2 mL of 50 mmol/L acetate–ammonium acetate buffer (pH 4.6), and 0.08 mL of approximately diluted culture filtrate was incubated at 40 °C for 30 min. The reaction was stopped by adding 8 μL of 200 g/L NaOH. The amount of glucose released in the reaction mixture was measured by employing the colorimetric method at 540 nm using 3,5-dinitrosalicylic acid reagent (DNS) (Miller, 1959). The blank was prepared using the culture filtrate after inactivation of the enzyme by NaOH. One unit (U) of glucoamylase activity was defined as the amount of enzyme that liberated 1 mg glucose per hour under the assay conditions.

2.7. Analysis of fumaric acid and sugars

The fumaric acid was determined and quantified by high performance liquid chromatography as previously reported (Fu et al., 2009). The concentration of the reducing sugars was measured by DNS method (Miller, 1959). The total reducing sugar was assayed by the same method after acid hydrolysis (6 mol/L HCl for 120 min at 100 °C) (Miller, 1959). The glucose concentrations were measured by SBA-80C biosensor analyzer (Institute of Biology, Shandong Academy of Sciences, China).

3. Results and discussion

3.1. The property of the strain ME-F12

SSF and SSF without the addition of glucoamylase were compared with respect to fumaric acid production using different carbon sources, such as cornstarch, corn powder, and degermed corn powder. The results are summarized in Table 1. Up to 32.57 g/L, 24.35 g/L, and 31.79 g/L of fumaric acid were obtained with productivities of 0.50 g/(L h), 0.34 g/(L h), and 0.44 g/(L h) in SSF when the initial total sugar concentrations of cornstarch, corn powder, and degermed corn powder were 80 g/L, 100 g/L, and 100 g/L. Lower concentrations (29.10 g/L, 17.50 g/L, 20.12 g/L) and