Direct Fermentation of Gelatinized Sago Starch to Acetone-Butanol-Ethanol by *Clostridium acetobutylicum*

Arbakariya Bin Ariff, Madihah Mohd Salleh, Suraini Abd Aziz and Mohamed Ismail Abdul Karim

Faculty of Food Science and Biotechnology
Institute of Bioscience
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor
Malaysia

Telephone Number of Corresponding Author: 03-86566427/89468342
E-mail of Corresponding Author: arbarif@putra.upm.edu.my

Key words: Solvent, sago starch, *Clostridium acetobutylicum*, chemostat culture and direct fermentation

Introduction

The main constraint on the economic viability of solvent (acetone-butanol-ethanol) fermentation is the cost of raw materials. Starch, the most abundant utilisable resource in plant biomass, can be hydrolysed enzymatically into fermentable sugars for subsequent use in acetone, ethanol and butanol (ABE) fermentation. However, this two step process is not very attractive due to high operating cost. Direct utilisation of starch by employing starch-fermenting saccharolytica *Clostridium* species for solvent production has been reported and this may reduce production cost by about 60-70%. The ability of *Clostridium* sp. to produce amylolytic enzymes such as amylases, pullulanase and glucoamylase enables direct fermentation of gelatinised starch to ABE. Utilisation of various types of starchy materials for ABE production by *Clostridium acetobutylicum* has been examined by several researchers. The feasibility of using potato starch as substrate in ABE fermentation has been studied intensively by several researchers. Malaysia produces an abundant supply of sago starch and it is normally used for the production of glucose. Glucose obtained from sago starch is used as a substrate for the fermentation industries and also for the production of high fructose syrup. However, direct fermentation of sago starch to valuable products has not fully been explored by the industry.

The present study was carried out to investigate the effect of medium formulation on performance of direct fermentation of sago starch to solvent by *C. acetobutylicum* P262. The effect of different process control strategies and mode of fermenter operation on the performance of solvent fermentation using gelatinised starch as the sole carbon source is also reported here.

Materials and Methods

The *C. acetobutylicum* P262 strain utilised in this study was maintained in a sterile RCM (Reinforced Clostridia Medium, Oxoid) medium and stored under anaerobic condition at 4°C. The culture medium containing the following components per L of distilled water; 50 g sago starch, 0.75g KH_2PO_4, 0.75 g K_2HPO_4, 0.4 g $MgSO_4.7H_2O$, 0.01 g $MnSO_4 H_2O$, 0.01 g $FeSO_4 H_2O$, 5.0 g yeast extract, 2.0 g NH_4NO_3, 0.5 g cystein and 1mg resazurin, was used in all experiments. The medium was sterilised at 121 °C for 20 minutes. A 4 mL filter sterilised solution containing 1 mg/L P-amino benzoic acid and 0.08 mg/L Biotin was also added to the sterilised medium.

Solvent fermentations were carried out using 250 mL Schott bottles and 2 L stirred tank fermenter (Chemap AG, Denmark). The fermenter was equipped with temperature and pH control systems, and operated as batch and chemostat (single-stage and two stage) cultures. The pH control system consisted of a standard PI controller and dead band control with two pumps for acid and alkali. The pH was controlled either by adding 3 N NaOH or 1 N HCl. A six-bladed turbine impeller (diameter = 50 mm) mounted on the agitator shaft was used for agitation. Sterile medium in Schott bottle or in the fermenter was kept under anaerobic conditions by sparging a nitrogen free oxygen gas before and after inoculation. A 10 % (v/v) inoculum was used to inoculate 1.5 L medium in the fermenter. In all fermentations, the temperature was controlled at 35 °C and agitation speed was fixed at 100 rpm (impeller tip speed = πND = m/s).

During the fermentation, samples were withdrawn at time intervals for analysis. The cell concentration was determined using oven dry method. Concentration of solvent was determined using gas chromatography (Shimadzu 17-A, Japan) equipped with flame ionisation detector (FID). On the other hand, organic acid (butyric and acetic acids) was determined by using high performance liquid chromatography with UV detector at 210 nm using HPX-87H (70 x 7.8H) Biorad column and 7 mM H_2SO_4 as a stationary and mobile phase, respectively. The profile of enzymes related to ABE fermentation and amylolytic enzymes (α-amylase and glucoamylase) were assayed using the standard method as described elsewhere.

Results and Discussion

The highest total solvent production (11.87 g/l) by *C. acetobutylicum* was obtained when corn starch was used. Total concentration of solvent obtained in fermentation using sago starch was comparable to that obtained by fermentation of corn starch. However, the overall productivity for fermentation using sago starch (0.26 g/l.h) was about 44% higher than fermentation using corn starch. Total solvent production in fermentation of tapioca and potato starches was about 2 times lower compared to fermentation using corn and sago starches. It is interesting to note that solvent yield, based on potential glucose present in the culture, in fermentation using sago starch was about 22% higher than fermentation using glucose. During the early stages of growth, sago starch was hydrolysed into maltose and glucose by α-amylase and glucoamylase enzymes. Activity of glucoamylase increased gradually and reached a maximum (2.76 U/mL) at 90 h of fermentation. On the

other hand, α-amylase activity increased rapidly and reached a maximum (1.98 U/mL) after 15 h. Direct fermentation of sago starch to solvent by *C. acetobutylicum* can be divided into two phases, acidogenic phase and solventogenic phase. An acidogenic phase was observed during the first 24 h of fermentation where *C. acetobutylicum* grew rapidly with production of organic acids (acetic and butyric acids) which caused reduction in culture pH from 6 to 4.5. The fermentation entered solventogenic phase when growth reached a stationary phase (i.e., after 50 h). During this phase, the metabolism of cells undergoes a shift to produce solvent by reassimilation of organic acid.

The performance of solvent fermentation by *C. acetobutylicum* was greatly influenced by sago starch concentration. Total solvent production was increased drastically from 10 to 50 g/L starch and a decrease in the production was observed at >60 g/L starch. Gelatinised sago starch exhibited pseudoplastic behaviour and its apparent viscosity increased drastically at >60 g/l starch. Reduced fermentation performance with high starch concentrations may be due to an increase in apparent viscosity, which in turn, limits the mass transfer for enzymatic hydrolysis and microbial reactions.

Higher solvent production was obtained in fermentation with the addition of inorganic nitrogen source to yeast extract as compared to fermentation using yeast extract alone. In addition, the type of inorganic nitrogen source used greatly influenced solvent production. The highest total solvent production (18.78 g/L) was obtained when NH_4NO_3 was used in a mixture with yeast extract and this was about 4.5 times higher than fermentation using yeast extract alone. Production of total solvent, increased drastically with increasing nitrogen concentration from 42.8 mM to 128.5 mM. The highest overall productivity (0.53 g solvent/L.h) was obtained at 256.9 mM nitrogen. On the other hand, the highest yield (2.07 g solvent/g initial nitrogen) was obtained at a lower nitrogen concentration (128.5 mM).

The optimal pH control strategy for improvement of solvent production was to let the pH regulated naturally during acidogenic phase (initial pH was 6) and pH was controlled at 5.5 during solventogenic phase. This optimal pH control strategy was also tested in fermentation using high sago starch concentrations (60–80 g/L). Among the range of starch concentration investigated, the highest solvent production (24.9 g/L), which gave an overall productivity of 0.77 g/L.h, was obtained at 70 g/L.

Culture pH and dilution rate (D) seemed to play important role in the performance of two stage continuous culture for solvent production. The highest solvent production (10.48 g/L), which gave and overall productivity of 0.39 g/L.h, could be achieved by maintaining the pH at 4.5 for both fermenter stages, while the continuous operation was operated at dilution rate of 0.08 h^{-1} and 0.037 h^{-1} for the first and second stage fermenter, respectively. However, solvent concentration in the outflow of the second stage fermenter was slightly lower than that obtained in a single stage continuous culture (11.34 g/L) and about 1.5 times lower than conventional batch culture (16.34 g/L). From enzyme analysis data, it was found that the specific activity of crotonase, thiolase, phosphate butyryl transferase and β-hydroxyl-CoA dehydrogenase were about 30% higher in solvent-producing cells than in acid-producing cells. However, crotonase was found to be the only enzyme that has high contribution towards solvent production as indicated by its highest activity (430.15 U/mg) at conditions (pH 4.5 and low D) where the highest production was achieved. The specific activity of butyryl CoA dehydrogenase was not detected during the fermentation.

Conclusions

Total solvent production from fermentation using sago starch was comparable to fermentation using corn starch and about two times higher than fermentation using potato or tapioca starch. At the range of sago starch concentration investigated (10 to 80 g/L), the highest total solvent production (18.82 g/L) was obtained at 50 g/L. Substantial improvement of direct fermentation of sago starch to solvent was achieved in fermentation where pH was not controlled during acidogenic phase (initial culture pH was 6) and then the pH was controlled at 5.5 during solventogenic phase. Using this pH control strategy, the overall productivity (0.79 g/L.h) was improved by 1.6 times as compared to fermentation without pH control, though the final solvent concentration was about the same. The use of chemostat culture, either a single or two stage, did not show significant improvement as compared conventional batch process.

Benefits from the study

Efficient anaerobic fermentation process using locally cheap and abundant local sources for solvent fermentation has been developed. Kinetics and models for the solvent fermentation by *C. acetobutylicum* using sago starch as substrate have been identified, proposed and described. Process arrangement for direct fermentation of sago starch to solvent that may be applied industrially has been proposed. The technology could be coupled with treatment of waste from food and agricultural sector for production of bio-energy. This is a strategic and important future technology for the country and the process is environmental friendly. Small scale production for laboratory use is being carried out at UPM.

Patent(s), if applicable:

Nil

Stage of Commercialization, if applicable:

Nil

Project Publications in Refereed Journals

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Project Publications in Conference Proceedings:
Nil

Graduate Research

Name of Graduate	Research Topic	Field of Expertise	Degree Awarded	Graduation Year
Madihad Md Salleh	Direct fermentation of sago starch to solvent (acetone-butanol-ethanol) by *Clostridium acetobutylicum* P262	Fermentation Technology	PhD	2001
Norsuhaila Yaacob	Performance of solvent (acetone-butanol-ethanol) production by *Clostridium saccharobutylicum* P262 and NCIMB 8052 using free and immobilised cell system	Fermentation Technology	MSc	2003

IRPA Project number: 09-02-04-0087
Project Leader Assoc. Prof. Dr. Arbakariya Bin Ariff
UPM Research Cluster: BAB