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Butanol Production by a Novel Efficient Method Using Mixed Cultures of *Clostridium beijerinckii* and *Arthrobacter* sp. in Stirred-Tank and Gas-Lift Bioreactors

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Abstract: *Arthrobacter* sp. BCC 72131, an obligate aerobic bacterium, was used to create anaerobic conditions, and *Clostridium beijerinckii* TISTR 1461 was used as a butanol producer in an acetone-butanol–ethanol (ABE) fermentation. Sweet sorghum juice (SSJ) medium containing 60 g/L of total sugar supplemented with 1.27 g/L of $(NH_4)_2SO_4$ was used as a butanol production (BP) medium. *Arthrobacter* sp. was inoculated into the BP medium in 1-L screw-capped bottles. After 2, 4, 6 and 12 h of *Arthrobacter* sp. cultivation at 30 °C, *C. beijerinckii* was transferred into the BP medium to start butanol production at 37 °C. The results showed that *C. beijerinckii* inoculation after 6 h of *Arthrobacter* sp. cultivation gave the highest butanol titer (P_B) at 12.56 g/L, with a butanol yield ($Y_{B/S}$) and volumetric butanol productivity (Q_B) of 0.34 g/g and 0.23 g/L·h, respectively. These values are approximately 10–27% higher than those of the control experiment using a single culture of *C. beijerinckii* TISTR 1461 and oxygen-free nitrogen (OFN) gas flushing to create anaerobic conditions. Field emission scanning electron microscopic (FE-SEM) images of *Clostridium* cells, as well as protein and free amino nitrogen concentrations in the broth during butanol fermentation were also studied to confirm the results. The butanol fermentation was then carried out in a 5.6-L stirred-tank and a 1.2-L low-cost gas-lift bioreactor by the mixed cultures using the optimal time of *Clostridium* inoculation. The P_B, $Y_{B/S}$ and Q_B values obtained were not significantly different from those in the 1-L screw-capped bottles. Hence, *Arthrobacter* sp. can be used as a novel method to create anaerobic conditions instead of a traditional method employing OFN gas flushing. Using mixed cultures of *Arthrobacter* sp. BCC 72131 and *C. beijerinckii* TISTR 1461 is a practical method to produce butanol on a large-scale, both in complex and low-cost bioreactors.

Keywords: anaerobic condition creation; *Arthrobacter* sp.; butanol production; *Clostridium beijerinckii*; sweet sorghum juice; gas-lift bioreactor; stirred-tank bioreactor; field emission scanning electron microscopy (FE-SEM)

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1. Introduction

Butanol is widely used as a chemical starting material for making plastics, plasticizers, polymers, paints, lubricants, brake fluids and synthetic rubber [1,2]. Butanol has a high energy density of 29.2 MJ/L and can be substituted for gasoline (energy density of 32 MJ/L) with no modifications to the current internal combustion engines [3]. Presently, butanol is most often obtained from crude oil [4]. However, with the increasing fluctuations in petroleum oil availability and costs as well accumulating environmental dilemmas, butanol fermentations are alternatives of renewed interest. Additionally, it is an excellent renewable fuel that can be considered a better transportation fuel than bioethanol in future fuel systems [5], mainly due to its higher number of carbon atoms and consequently higher energy content, miscibility with gasoline and less corrosivity [6]. Additionally,

the strong renewed attention given to butanol as a sustainable vehicle fuel has induced progress in improving biobutanol production by developing biotechnological production and separation processes [1].

Biobutanol can be produced by *Clostridia* strains via an ABE fermentation under strictly anaerobic conditions. In batch ABE fermentations, biphasic metabolism is observed. *Clostridium* sp. produces hydrogen, carbon dioxide, acetate and butyrate during the initial growth phase in an acidogenic phase, resulting in a decreased pH of the culture medium. Cell metabolism then shifts to solvent production to form acetone, butanol and ethanol in a solventogenic phase [7–9].

Oxygen-free nitrogen (OFN) gas is generally used to create anaerobic conditions for ABE fermentations at a laboratory scale [10]. It is, however, impractical in larger bioreactors because of the high operational costs and complexity of applying OFN gas. Our preliminary research found that an obligate aerobic bacterium, *Arthrobacter* sp., could consume oxygen in a bioreactor and create anaerobic conditions for ABE fermentation by *Clostridium* sp. [11]. Nevertheless, the optimum parameters for using this anaerobic bacterium to create anaerobic conditions for butanol fermentation by *C. beijerinckii* TISTR 1461 have not been reported.

Sweet sorghum is a non-competitive substrate that can be cultivated with lower water requirements and is tolerant to salinity and drought. It can be harvested 3–4 times per year. Sweet sorghum stalks contain up to 78% juice [12]. The juice from its stalks consists of fermentable sugars (sucrose, glucose and fructose) that can be directly fermented to produce acetone, butanol and ethanol by *Clostridium* spp. Daengbussadee et al. [11] reported that a simplified SSJ medium (60 g/L of sugar) supplemented with only 1.27 g/L of $(NH_4)_2SO_4$ could be used as a butanol production medium yielding a product concentration (P_B) of 9.88 g/L.

Laboratory-scale bioreactors, screw-capped bottles, air-locked flasks and stirred-tank bioreactors are generally used to study ABE fermentations. Mixing, mass transfer and heat transfer are completely obtained using a magnetic stirring bar in screw-capped bottles and air-locked flasks. In larger vessels, they are entirely achieved by mechanical agitation with impellers and baffles in stirred-tank bioreactors. However, the fabrication and operation of stirred tank bioreactors are costly. Such reactors have many accessories, are complex in their operation and are difficult to clean and set up. Thus, a low-cost gas-lift bioreactor used as a tower bioreactor for large-scale production was applied in the current study to evaluate butanol production.

Most earlier research in mixed cultures for ABE fermentations was conducted using *Clostridium* spp. and facultative aerobic bacteria (*Bacillus* spp.) [13–17]. This study was performed to develop an effective technique to create anaerobic conditions by cultivating the obligate aerobic bacterium, *Arthrobacter* sp. BCC 72131, prior to ABE fermentation by *C. beijerinckii* TISTR 1461—which has never been reported. The optimum conditions of ABE fermentation for the mixed cultures were determined. Butanol production by the mixed cultures using a low-cost bioreactor (a gas-lift bioreactor) was first used to appraise its butanol production efficiency compared to using a complex bioreactor (a stirred-tank bioreactor). Additionally, the relationships between cell morphology and product formation during an ABE fermentation by mixed cultures were first reported in this paper.

2. Materials and Methods

2.1. Raw Materials

Sweet sorghum juice (SSJ) (cv. KKU 40) extracted from sorghum stalks was obtained from the Faculty of Agriculture, Khon Kaen University, Thailand. It was concentrated from a total soluble solids content of 17 to 68° Bx by heating at 80–85 °C and stored at −20 °C to protect it against bacterial growth [18]. The composition of concentrated SSJ in this study consisted of total soluble sugars (677.02 g/L), sucrose (236.63 g/L), glucose (201.61 g/L), and fructose (233.14 g/L).

2.2. Butanol Production (BP) Medium Preparation

Concentrated SSJ was diluted with distilled water to obtain a juice containing 60 g/L of total sugars. The juice was then supplemented with 1.27 g/L of $(NH_4)_2SO_4$ (BDH, Poole, UK) and autoclaved before use as a butanol production (BP) medium [11].

2.3. Microorganisms and Inoculum Preparation

C. beijerinckii TISTR 1461 was purchased from the Thailand Institute of Scientific and Technology Research (TISTR), Khlong Luang, Pathumthani, Thailand. It was maintained as a spore suspension and stored at 4 °C in sterile distilled water. One milliliter of the spore suspension (~1×10^6 spores/mL) was activated by heat shocking and rejuvenating under anaerobic conditions in a sterile cooked meat medium (CMM) (Oxoid, Basingstoke, Hants, UK) at 37 °C for 16–19 h. The vegetative cells (5% *v*/*v*) in CMM medium were inoculated into sterile tryptone–glucose–yeast extract (TGY) medium and incubated at 37 °C for 4–6 h before use as an inoculum for the ABE fermentation [11,19,20]. TGY medium is comprised of 5 g tryptone (Oxoid, Basingstoke, Hants, UK), 1 g glucose (BDH, Leuvn, Belgium), 5 g yeast extract (Oxoid, Basingstoke, Hants, UK) and K_2HPO_4 (BDH, Leuvn, Belgium) in 1 L distilled water. The CMM and TGY media were autoclaved and purged with OFN gas before use.

Arthrobacter sp. BCC 72131 was purchased from the Thailand Bioresource Research Center (TBRC), Khlong Luang, Pathumthani, Thailand. Cells were grown in a sterile nutrient broth (NB) under shaking conditions at 200 rpm and 30 °C for 6–7 h. Afterwards, the culture was transferred to fresh NB under the same conditions to obtain an optical density at 600 nm of 0.5 (~0.8 g/L of cell dry weight) [11] before use as an inoculum to consume oxygen in a fermentation broth and thereby create anaerobic conditions.

2.4. Experiments

2.4.1. Butanol Production by a Single Culture of *C. beijerinckii* TISTR 1461

For a positive control experiment, OFN gas at a flow rate of 0.5 vvm was purged for 10 min into a sterile BP medium (Section 2.2) in 1-L screw-capped bottles with a working volume of 700 mL to create anaerobic conditions before fermentation [21]. Five percent (*v*/*v*) of the active growing cells (Section 2.3) was then transferred to the BP medium. The fermentation was operated at 37 °C with an agitation rate of 150 rpm until the end of fermentation. For a negative control experiment, the fermentation was carried out in the same manner as the positive control experiment, except there was no OFN gas flushing.

2.4.2. Butanol Production by Mixed Cultures of *Arthrobacter* sp. BCC 72131 and *C. beijerinckii* TISTR 1461

First, 5% (*v*/*v*) of *Arthrobacter* sp. BCC 72131 in NB (Section 2.3) was inoculated in sterile BP medium in 1-L screw-capped bottles and incubated at 30 °C with 150 rpm for 2, 4, 6 and 12 h. After each time, the active growing cells of *C. beijerinckii* TISTR 1461 were transferred to a BP medium to start the ABE fermentation. The fermentation was controlled at 37 °C with agitation at 150 rpm.

2.4.3. Butanol Production by Mixed Cultures in Stirred-Tank and Gas-Lift Bioreactors

A 5.6-L stirred-tank bioreactor (BioFlo® 320 Vessels, Eppendorf, Hamburg, Germany) and a 1.2-L low-cost gas-lift bioreactor, shown in Figure 1, were sterilized before adding a sterile BP medium with working volumes of 3.6 and 0.8 L, respectively. *Arthrobacter* sp. BCC 72131 (5% *v*/*v*) was then inoculated into the sterile BP medium in both bioreactors and incubated at 30 °C with agitation rate of 150 rpm using a six-blade dish turbine and four baffles in the stirred-tank bioreactor. Mixing in the gas-lift bioreactor was controlled with a draft tube and the circulation of gases in the head space was achieved using a peristaltic pump (Masterflex®, Radnor, PA, USA) at a flow rate of 0.25 vvm (modified from [22]). After the optimum time of *Arthrobacter* cultivation (obtained as described in Section 2.4.2), active *C. beijerinckii* TISTR 1461 cells were added into the bioreactors to start ABE fermentations.

Butanol fermentations were performed at 37 °C. During fermentation, samples were taken at predetermined time intervals for analyses.

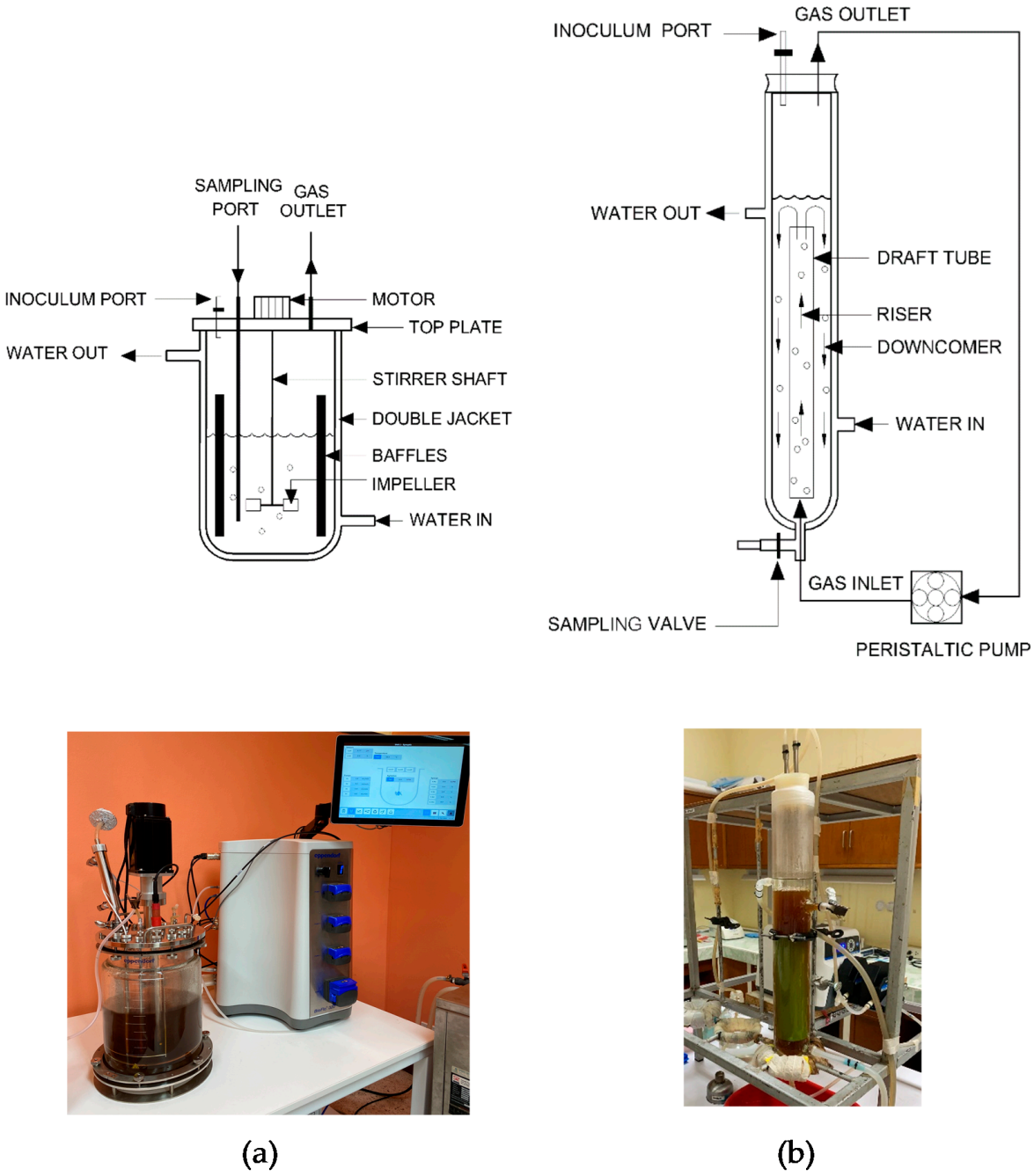

Figure 1. Experimental setup for butanol fermentation in (**a**) stirred-tank bioreactor and (**b**) gas-lift bioreactor.

2.5. Analytical Methods

Samples were centrifuged at 12,000 rpm for 10 min to the separate cells and particles from the supernatant (Sigma 1–14, Sartorius, Osterode am Harz, Germany). The supernatant was filtered through a 0.45 µm nylon membrane (Sartorius, Goettingen, Germany) for analysis. Acetone, butanol, ethanol, acetic acid, and butyric acid in the supernatant were analyzed using a gas chromatograph (Shimadzu, GC-2014, Kyoto, Japan) employing a Porapak Q column (80/100 mesh, 3 m × 2 mm, Resteck, PA, USA), and nitrogen was used as a carrier gas [11]. pH was measured using a pH meter. The total sugar concentration was

determined using a phenol–sulfuric acid method [23]. A quantitative analysis of fructose, glucose and sucrose was performed with an HPLC-RI (Waters, MA, USA) using an Inertsil® NH2 column (5 µm, 250 × 4.6 mm, GL Sciences, Tokyo, Japan). The analysis was performed at 35 °C with a flow rate of 0.8 mL/min using an isocratic system with a mixture ratio of 75 parts acetonitrile: 25 parts water (*v*/*v*) as a mobile phase [24,25]. Protein and free amino nitrogen (FAN) concentrations under optimal conditions and control experiments were also measured using the Bradford method [26] and a ninhydrin assay [27], respectively.

The morphology of *C. beijerinckii* TISTR 1461 during fermentation was observed using field emission scanning electron microscopic (FE-SEM) analysis. Samples were taken from the bioreactors, quickly filtered through a 0.2 µm cellulose acetate membrane (Whatman®, Maidstone, England), and washed twice with distilled water. The filtrate and bacterial cells remaining on the membrane were then dried overnight at 50 °C in a hot air oven. Then, the dried material was sputter-coated with gold. An accelerating voltage of 10 kV under a vacuum was used to yield high-resolution images. Microscopic analysis was performed with a FEI Helios Nanolab G3 CX Dual Beam FIB/SEM, Thermo Fisher Scientific, Hillsboro, OR, USA.

All experiments were performed in triplicate, and the results are expressed as mean values ± SD. The butanol yield ($Y_{B/S}$, g/g), ABE yield ($Y_{ABE/S}$, g/g), volumetric butanol productivity (Q_B, g/L·h) and sugar consumption (*SC*, %) were calculated as described in Daengbussadee et al. [11].

3. Results and Discussion

3.1. Butanol Production from SSJ Medium by Single and Mixed Cultures

Butanol fermentations by a single culture of *C. beijerinckii* TISTR 1461 with and without OFN gas flushing were carried out as positive and negative control treatments, respectively. The pH changes during the fermentation of both control treatments were similar (Figure 2a). However, the P_B (9.88 ± 0.38 g/L), ABE concentrations (P_{ABE}, 17.61 ± 0.63 g/L) and sugar utilization (53.46 ± 0.46%) were clearly higher for the positive control with OFN gas flushing than those of the negative control with no OFN gas flushing (Figure 2b–d and Table 1). This confirms that *C. beijerinckii* TISTR 1461 produced higher butanol levels under strictly anaerobic conditions.

For mixed cultures, after 2, 4, 6 and 12 h of *Arthrobacter* sp. cultivation, *C. beijerinckii* TISTR 1461 was then inoculated to start the ABE fermentation. The results showed that the ABE fermentation profiles of all conditions of the mixed cultures were similar to those of the positive control treatment using OFN gas flushing (Figure 2). The P_B, sugar utilization and Q_B of the conditions under which *C. beijerinckii* TISTR 1461 was inoculated after 2 and 4 h were not significantly different compared to the positive control treatment (Table 1). These results clearly indicated that an obligate aerobe, *Arthrobacter* sp., could be used to create anaerobic conditions. The inoculation of *C. beijerinckii* TISTR 1461 after 6 h gave the highest P_B (12.56 ± 0.55 g/L) and P_{ABE} (21.18 ± 0.44 g/L). Surprisingly, the P_B and P_{ABE} of the mixed cultures under this condition were approximately 20–27% higher than those of the positive control treatment. The P_B when *C. beijerinckii* was inoculated after 12 of *Arthrobacter* sp. cultivation was not significantly different from inoculation after 6 h (Table 1). This suggests that 6 h of *Arthrobacter* sp. cultivation was appropriate for developing anaerobic conditions for butanol production by *C. beijerinckii* TISTR 1461. The P_{ABE} value when *C. beijerinckii* was inoculated after 12 h of *Arthrobacter* sp. cultivation was lower than that after 6 h, but the sugar utilization of the former was higher than the latter. These observations might be due to the differences in the by-products produced by the pyruvate metabolism pathway that were not detected in this study such as hydrogen, isopropanol, lactic and propionic acids, carbon dioxide and lipids [28].

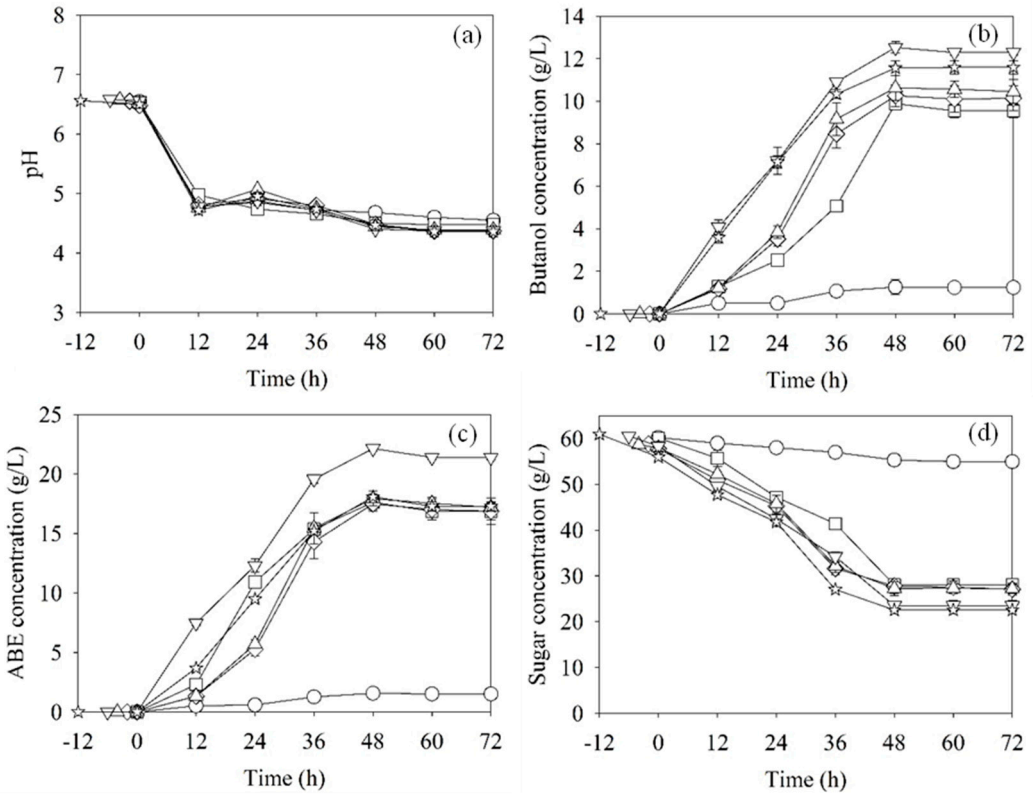

Figure 2. Profiles of pH (**a**), butanol (**b**), ABE (**c**) and sugar (**d**) concentrations during ABE fermentation by the mixed cultures and single culture (positive control (□); negative control (○)). In the mixed cultures, *C. beijerinckii* TISTR 1461 was inoculated after 2 (◇), 4 (△), 6 (▽) and 12 h (☆) of *Arthrobacter* sp. cultivation.

Table 1. Kinetic parameters of ABE fermentation by the mixed cultures of *C. beijerinckii* TISTR 1461 and *Arthrobacter* sp. BCC 72131 at different times of *Arthrobacter* sp. cultivation prior to *C. beijerinckii* inoculation and by a single culture of *C. beijerinckii* TISTR 1461 using OFN gas flushing.

Condition	P_B (g/L)	P_{ABE} (g/L)	P_{acid} (g/L)	*SC* (%)	*t* (h) *	$Y_{B/S}$ (g/g)	Q_B (g/L·h)
Mixed cultures involving *Arthrobacter* sp. cultivation prior to *C. beijerinckii* inoculation for							
2 h	10.25 ± 0.31^{b}	17.49 ± 0.39^{b}	1.75 ± 0.46^{bc}	54.84 ± 0.21^{c}	50	0.31 ± 0.00^{b}	0.21 ± 0.01^{b}
4 h	10.62 ± 0.37^{b}	17.92 ± 0.32^{b}	1.30 ± 0.05^{c}	55.84 ± 0.21^{c}	52	0.33 ± 0.01^{a}	0.20 ± 0.01^{bc}
6 h	12.56 ± 0.55^{a}	21.18 ± 0.44^{a}	1.84 ± 0.25^{b}	60.44 ± 0.09^{b}	54	0.34 ± 0.00^{a}	0.23 ± 0.00^{a}
12 h	11.81 ± 0.56^{a}	18.08 ± 0.20^{b}	1.90 ± 0.18^{b}	62.04 ± 0.52^{a}	60	0.31 ± 0.00^{b}	0.19 ± 0.01^{c}
Single culture of *C. beijerinckii* TISTR 1461							
: OFN flushing (Positive control)	9.88 ± 0.38^{b}	17.61 ± 0.63^{b}	1.54 ± 0.21^{bc}	53.46 ± 0.46^{d}	48	0.30 ± 0.01^{b}	0.21 ± 0.01^{b}
: No OFN flushing (Negative control)	1.26 ± 0.35^{c}	1.56 ± 0.12^{c}	4.22 ± 0.02^{a}	7.90 ± 0.56^{e}	48	0.31 ± 0.01^{b}	0.04 ± 0.00^{d}

* Fermentation time includes the time of *Arthrobacter* sp. cultivation. $^{a–e}$ Means followed by the same letter within the same column are not significantly different using Duncan's multiple range test at the level of 0.05. The results in the table were performed in at least triplicate experiments and the results expressed as mean values ± SD. P_B = butanol concentration; P_{ABE} = acetone–butanol–ethanol concentration; P_{acid} = total acid concentration; *SC* = sugar consumption; *t* = fermentation time; $Y_{B/S}$ = butanol yield; and Q_B = volumetric butanol productivity.

It was found that sugar consumption by *Arthrobacter* sp. before *C. beijerinckii* TISTR 1461 inoculation in all experiments ranged from 1.54 to 3.96 g/L. This implies that *Arthrobacter* sp. consumed some sugar and all the oxygen in the fermentation broth to grow or survive. During ABE fermentation, *C. beijerinckii* TISTR 1461 continuously consumed sugars in the fermentation broth. Under inoculating *C. beijerinckii* after 6 h of *Arthrobacter* sp. cultivation, the total remaining sugars at the end of fermentation were 24.42 g/L (~60%

sugar consumption). The residual sugar fraction consisted of glucose (0.40 g/L), fructose (8.90 g/L), sucrose (9.57 g/L) and non-fermentable sugars (5.55 g/L). The fermentable sugars in the broth were not completely consumed, which might have been due to butanol toxicity (P_B, 12.56 ± 0.55 g/L and P_{ABE}, 21.18 ± 0.44 g/L). This was supported by Xu et al. [29] who reported that the cell growth stopped when the butanol concentration reached ~10 g/L and the fermentation stopped at ~12 g/L of butanol, although there was still plenty of glucose present in the medium.

After 12 h of fermentation by the mixed cultures, the cell debris of *Arthrobacter* sp. was observed under a light microscope. Therefore, the protein and free amino nitrogen (FAN) in the fermentation broth were determined during the ABE fermentation (Figure 3). It was found that the initial values of protein and FAN in the SSJ medium using the mixed cultures and single cultures were not different. Protein and FAN increased within 24 h and decreased after that in the mixed cultures. In the fermentation by the single culture, the protein content in the broth was relatively constant and the FAN continuously decreased as the ABE fermentation progressed. In the fermentation by the mixed cultures, the increase in protein and FAN within 24 h after *C. beijerinckii* inoculation might have been due to the autolysis of *Arthrobacter* sp. This might promote the growth and butanol production of *C. beijerinckii* TISTR 1461 in a way that is not possible using a single culture of the *Clostridium*.

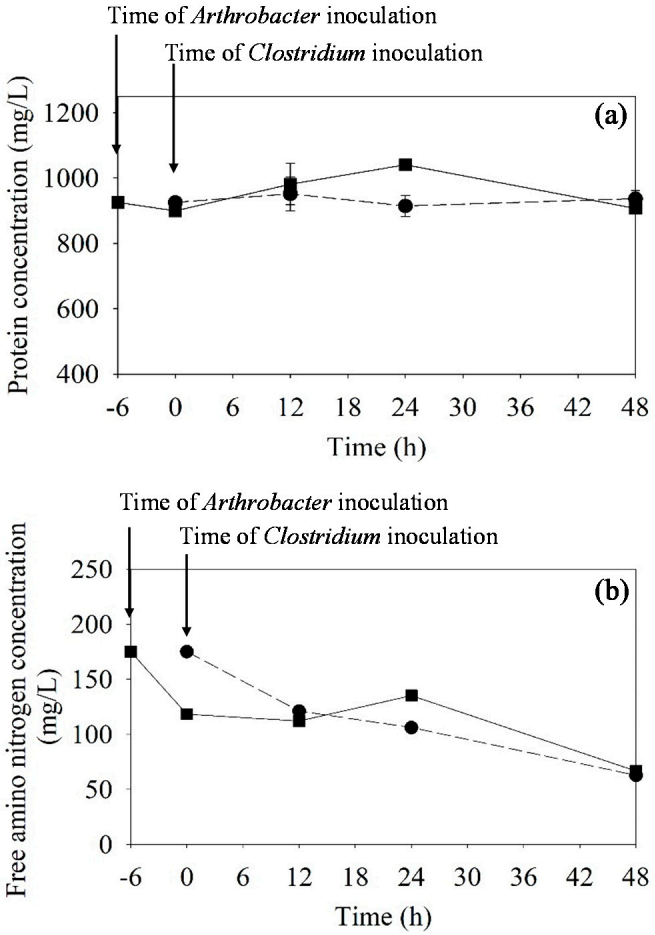

Figure 3. Profiles of protein (**a**) and free amino nitrogen (FAN) (**b**) concentrations in the fermentation broth during ABE fermentation by mixed cultures of *Arthrobacter* sp. and *C. beijerinckii* TISTR 1461 (inoculating *C. beijerinckii* after 6 h of *Arthrobacter* cultivation, ■) and by a single culture of *C. beijerinckii* using OFN gas flushing (●).

3.2. Morphology of C. beijerinckii TISTR 1461 during ABE Fermentation by a Single Culture and Mixed Cultures

The morphologies of *C. beijerinckii* TISTR 1461 during ABE fermentation by single and mixed cultures under optimal conditions were compared. The FE-SEM micrographs of a single culture using OFN gas flushing are shown in Figure 4. In the first phase—the acidogenesis phase (0–12 h) (Figure 2a)—the cells appeared as short and highly motile rods (Figure 4a,b). During 24–36 h of ABE fermentation, the cells were enlarged, swollen and moved sluggishly (Figure 4c,d). In this phase—the solventogenesis phase—high butanol levels were produced (Figure 2b). After that, forespores were observed (Figures 4e and 5a), and the maximal P_B value was achieved at 48 h of fermentation (Figure 2b). The occurrence of forespores might have been due to butanol toxicity. Then, no butanol was produced until the end of fermentation. During this period, the cells had two survival mechanisms: solventogenesis and sporulation (Figure 4f) [30].

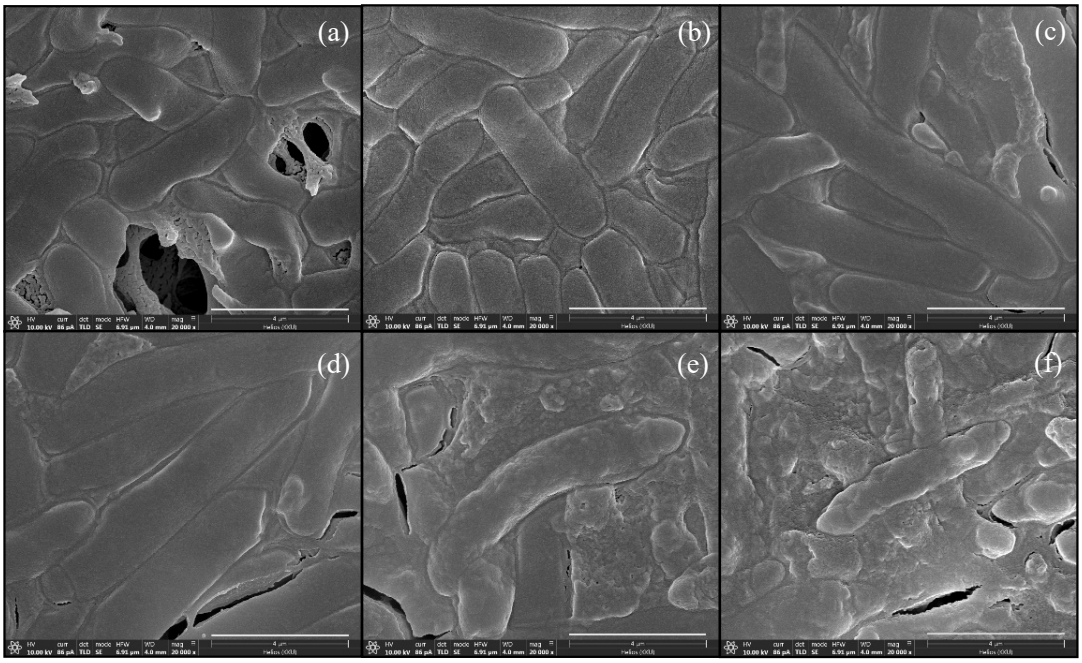

Figure 4. FE-SEM micrograph of *C. beijerinckii* TISTR 1461 during ABE fermentation in SSJ medium by a single culture at 0 h (**a**), 12 h (**b**), 24 h (**c**), 36 h (**d**), 48 h (**e**) and 60 h (**f**) of fermentation. Magnification of 20,000×. Scale bar: 4 µm.

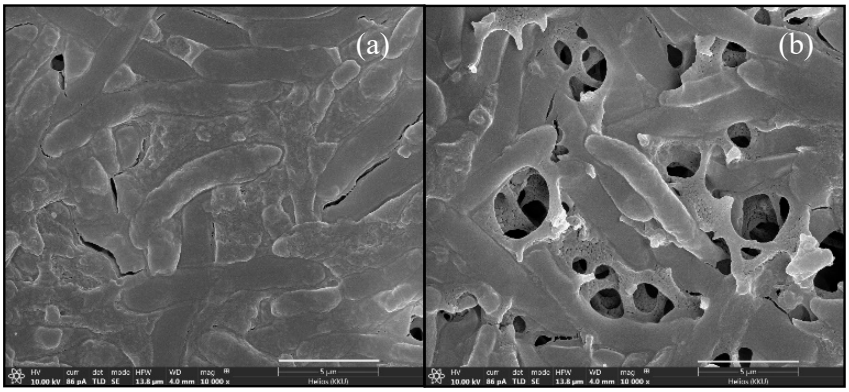

Figure 5. FE-SEM micrograph of *C. beijerinckii* TISTR 1461 at 48 h of ABE fermentation in SSJ medium by a single culture (**a**) and mixed cultures (inoculating *C. beijerinckii* after 6 h of *Arthrobacter* cultivation) (**b**). Magnification of 10,000×. Scale bar: 5 µm.

In ABE fermentations by the mixed cultures, the morphology of *C. beijerinckii* TISTR 1461 appeared perfect with strongly motile long rods (Figure 5b), and a higher P_B (12.56 ± 0.55 g/L) was obtained at 48 h after *C. beijerinckii* TISTR 1461 inoculation (Table 1). In contrast, the morphology of single culture *C. beijerinckii* TISTR 1461 at 48 h was imperfect and forespores were observed (Figure 5a). A lower P_B value (9.88 ± 0.38 g/L) was obtained in a single culture (Table 1). The results of FE-SEM indicated that *Arthrobacter* sp. could not only be used to effectively create anaerobic conditions, but its use also promoted butanol production from the SSJ medium by *C. beijerinckii* TISTR 1461.

3.3. Butanol Production by the Mixed Cultures in Stirred-Tank and Gas-Lift Bioreactors

To study the capability of butanol production by the mixed culture in a low-cost bioreactor, i.e., a gas-lift bioreactor, the fermentation profiles of butanol production from the SSJ medium by mixed cultures of *C. beijerinckii* TISTR 1461 and *Arthrobacter* sp. BCC 72131 (inoculating *C. beijerinckii* after 6 h of *Arthrobacter* cultivation) in the screw-capped bottle, stirred-tank and gas-lift bioreactors were compared in Figure 6. The pHs profiles in the three bioreactors were similar (Figure 6a). The pHs decrease corresponded to the production of acids (Figure 6b,d), indicating that bacterial cells were active. Moreover, the movement of cells during this period under the microscope was swift (data not shown). In a stirred-tank bioreactor, the sugar consumption and product formation during the first period of fermentation were slightly higher than those in the screw-capped bottle and gas-lift bioreactor (Figure 6e–i). These might be due to the positive effect of the mixing. The results suggested that mixing, mass transfer and heat transfer phenomena in the stirred-tank bioreactor occurred faster than those in the screw-capped bottle and gas-lift bioreactor. However, the P_B of each mixing method in screw-capped bottle (12.56 ± 0.55 g/L), stirred-tank bioreactor (12.59 ± 0.45 g/L) and gas-lift bioreactor (12.00 ± 0.48 g/L) were not significantly different at the end of fermentation (Table 2). The kinetic parameters of ABE fermentation in the various bioreactors tested were not significantly different, suggesting that the mass transfer phenomenon in the bioreactors successfully occurred via mixing. In addition, the ABE yield ($Y_{ABE/S}$) and $Y_{B/S}$ under all tested conditions was not significantly different, indicating that the types of bioreactor used did not affect the metabolic pathway of ABE fermentation by the mixed cultures (Table 2). With regard to the sizes of the bioreactors and the head space during ABE fermentation, the working volumes of the 1-L screw-capped bottle, 5.6-L stirred-tank bioreactor and 1.2-L gas-lift bioreactor were 0.7, 3.6 and 0.8 L, respectively. These correspond to head space volumes of 30, 36 and 33%, respectively. Similar ABE fermentation results in Table 2 imply that the different sizes of three bioreactors with a similar head space did not have any impact on ABE fermentation. Therefore, *Arthrobacter* sp., an obligate aerobe bacterium, is an effective microorganism for creating anaerobic conditions for butanol production by *C. beijerinckii* TISTR 1461 in all bioreactors, including the low-cost bioreactor, i.e., the gas-lift bioreactor.

3.4. Comparison of Butanol Production by Mixed Cultures with Other Studies

In this study, *Arthrobacter* sp. BCC 72131 was used to create anaerobic conditions for butanol production by *C. beijerinckii* TISTR 1461. Earlier research was performed using other microorganisms (e.g., *Bacillus subtilis*, *B. cereus*, *B. cellolyticus* and *Saccharomyces cerevisiae*) to create anaerobic conditions for butanol production by *Clostridium*. Butanol production levels by mixed cultures are summarized in Table 3. It was found that the P_B values in all previous research ranged from 3.77 to 14.90 g/L, while Q_B and $Y_{B/S}$ ranged from 0.02 to 0.23 g/L·h and from 0.21 to 0.34 g/g, respectively. The P_B values in the current study were relatively high compared to earlier works. In our study, the P_B was slightly lower than that reported by Abd-Alla and Elsadek El-Enany [15], which might have been due to the differences in initial substate concentration, the bacterial cells used and environmental conditions for ABE fermentation. However, Q_B and $Y_{B/S}$ in our study were higher than those in other studies by approximately 1.1–11.5-fold. This implies that *Arthrobacter* sp. has high potential for use to create anaerobic conditions for butanol production. Butanol

production by the mixed cultures of *Arthrobacter* sp. and *C. beijerinckii* TISTR 1461 has high potential for use in large-scale production.

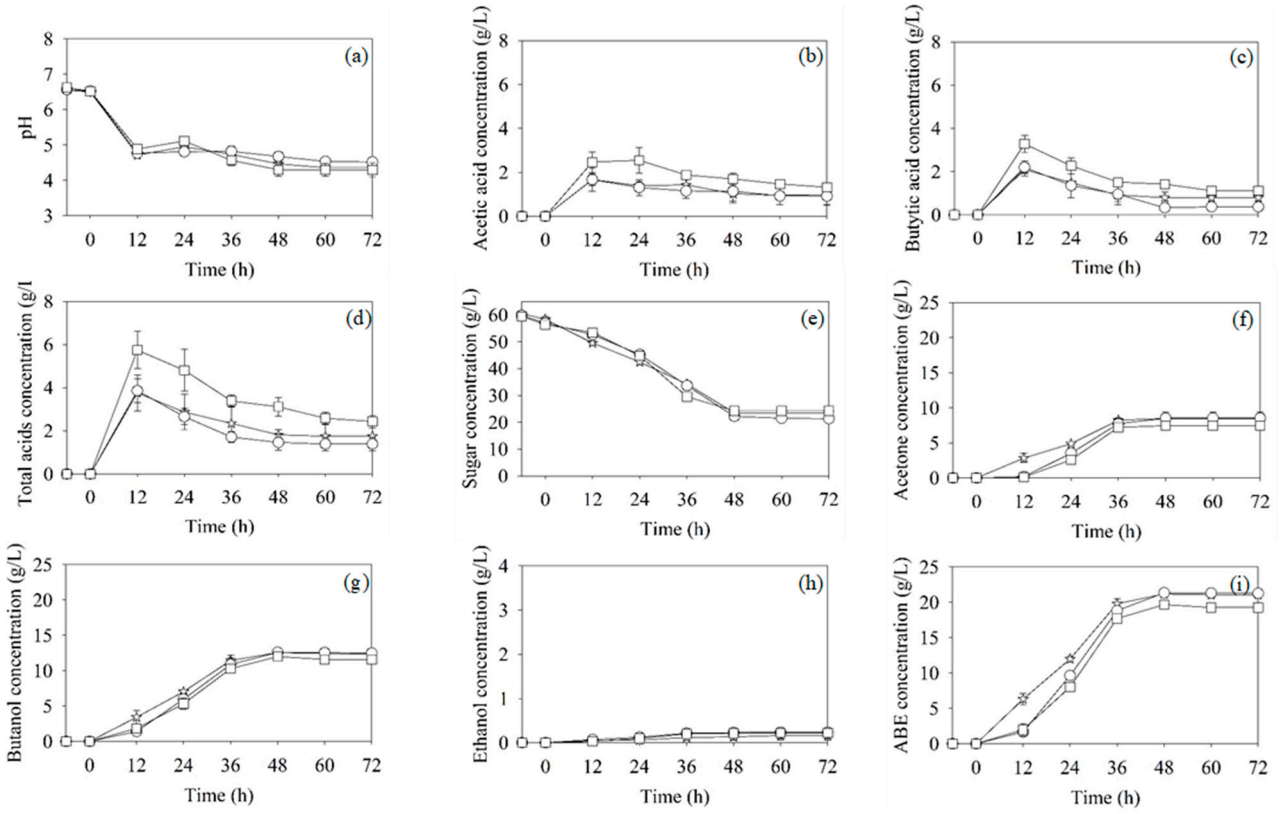

Figure 6. Profiles of pH (**a**), acetic acid (**b**), butyric acid (**c**), total acids (**d**), total sugar (**e**), acetone (**f**), butanol (**g**), ethanol (**h**) and ABE concentrations (**i**) during batch ABE fermentation by the mixed cultures in screw-capped bottles (○), stirred-tank bioreactor (☆) and gas-lift bioreactor (□).

Table 2. Kinetic parameters of ABE fermentation by the mixed cultures of *C. beijerinckii* TISTR 1461 and *Arthrobacter* sp. BCC 72131 (inoculating *C. beijerinckii* after 6 h of *Arthrobacter* cultivation) in various bioreactors after 54 h of fermentation.

Bioreactor	P_B (g/L)	P_{ABE} (g/L)	*SC* (%)	$Y_{B/S}$ (g/g)	Q_B (g/L·h)	$Y_{ABE/S}$ (g/g)
Screw-capped bottle	12.56 ± 0.55 [a]	21.18 ± 0.44 [a]	60.44 ± 0.09 [b]	0.34 ± 0.00 [a]	0.23 ± 0.00 [a]	0.60 ± 0.02 [a]
Stirred-tank bioreactor	12.59 ± 0.45 [a]	21.33 ± 0.54 [a]	62.57 ± 1.13 [a]	0.34 ± 0.00 [a]	0.23 ± 0.00 [a]	0.58 ± 0.02 [ab]
Gas-lift bioreactor	12.00 ± 0.48 [a]	19.68 ± 0.51 [b]	60.48 ± 0.82 [b]	0.33 ± 0.01 [a]	0.22 ± 0.01 [a]	0.56 ± 0.01 [b]

[a,b] Means followed by the same letter within the same column are not significantly different using Duncan's multiple range test at the level of 0.05. All experiments were performed in triplicate and the results are expressed as mean values ± SD. P_B = butanol concentration; P_{ABE} = acetone–butanol–ethanol concentration; *SC* = sugar consumption; $Y_{B/S}$ = butanol yield; Q_B = volumetric butanol productivity; and $Y_{ABE/S}$ = ABE yield.

An alternative method to create anaerobic conditions for ABE fermentations is using chemicals that strongly react with oxygen such as sodium dithionite (STDN). It was reported that 0.25 mM SDTN could be used to create anaerobic conditions for *C. beijerinckii* to produce butanol (8.51 g/L) but very low butanol productivity (0.1 g/L·h) was obtained [11]. According to our results, *Arthrobacter* sp. is suitable to be used to create anaerobic conditions for butanol production. During anaerobic condition creation, sugar consumption by *Arthrobacter* sp. in various bioreactors was only 2.20–2.76 g/L, corresponding to 3.67–4.60% of the total sugar. However, the economics of using the mixed cultures (*Arthrobacter* sp. and *C. beijerinckii* TISTR 1461) and a single culture of *C. beijerinckii* TISTR 1461 with OFN gas flushing needs to be further evaluated [31].

Table 3. Batch butanol production by mixed cultures in earlier research.

Substrate	Mixed Cultures	Bioreactor	Butanol Production			References
			P_B (g/L)	Q_B (g/L·h)	$Y_{B/S}$ (g/g)	
SSJ (60 g/L of sugar)	*C. beijerinckii* TISTR 1461 + *Arthrobacter* sp.	1-L screw-capped bottle	12.56	0.23	0.34	The current study
SSJ (60 g/L of sugar)	*C. beijerinckii* TISTR 1461 + *Arthrobacter* sp.	5.6-L stirred-tank bioreactor	12.59	0.23	0.34	The current study
SSJ (60 g/L of sugar)	*C. beijerinckii* TISTR 1461 + *Arthrobacter* sp.	1.2-L gas-lift bioreactor	12.00	0.22	0.33	The current study
Cassava starch (40 g/L of starch)	*C. butylicum* TISTR 1032 + *B. subtilis* WS 161	120-mL serum bottle	6.70	0.10	0.21	[13]
Pretreated palm pressed fiber (5 g/L)	*C. acetobutylicum* DSM 1731 + *B. cellolyticus* JCM 9156	60-mL serum bottle	3.77	0.02	-	[14]
Spoilage date fruit (75 g/L)	*C. acetobutylicum* ATCC 824 + *B. subtilis* DSM 4451	2-L fermenter	14.90	0.21	0.29	[15]
Corn mash (6.5% by wt. of corn flour)	*C. beijerinckii* NCIMB 8052 + *B. cereus* CGMCC 1.895	5-L fermenter	6.78	0.07	-	[16]
Agave hydrolysate (52 g/L)	*C. acetobutylicum* ATCC 824 + *B. subtilis* CDBB 555	120-mL glass bottle	8.28	0.10	0.29	[17]
Mixed-sugars (25 g/L of xylose and 25 g/L of glucose)	*C. acetobutylicum* CH02 + *S. cerevisiae*	250-mL flask	8.33	0.09	0.18	[32]

P_B, butanol concentration; Q_B, volumetric butanol productivity; and $Y_{B/S}$, butanol yield.

4. Conclusions

Mixed cultures of *Arthrobacter* sp. BCC 72131 and *C. beijerinckii* TISTR 1461 were successfully used to produce butanol from a simple sweet sorghum juice medium. *Arthrobacter* sp. is a highly effective bacterium for creating anaerobic conditions that can be used in place of the traditional method employing OFN gas flushing. Inoculating *C. beijerinckii* 6 h after *Arthrobacter* sp. was the most appropriate method for butanol production from sweet sorghum juice. The mixed cultures yielded higher butanol production than a single culture of *C. beijerinckii* TISTR 1461. Butanol production by mixed cultures of *Arthrobacter* sp. BCC 72131 and *C. beijerinckii* TISTR 1461 can be applied in large-scale stirred-tank and low-cost gas-lift bioreactors.

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