Short Communication

Hydrogen oxidizing bacteria as poly(hydroxybutyrate) producers

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Abstract

Batch culture of *Ralstonia eutropha* using the recycled gas closed circuit culture system was conducted in order to develop a practical fermentation system for industrial autotrophic culture for poly (hydroxybutrate) production. The gas phase of the culture system consisted of substrate gas so that gases in this culture could be recycled as long as the amount of the gas consumed would be replenished. All gases supplied into this system could be completely used without any loss as exhaust. Studies on the effect of oxygen concentration showed that high concentration of this gas suppressed the specific growth rate while a low oxygen concentration promoted it.

Keywords: Chemolithoautotroph; Gaseous substrate; Hydrogen oxidizing bacteria; Poly (β-hydroxybutyrate); Ralstonia eutropha.

The approach to tackle the greenhouse effect due to CO_2 pollution in atmosphere is very urgent for mankind and a feasible technology has not yet been established. The culture of hydrogen oxidizing bacteria (HOB) is a matter worth considering as a possible tool. Although the autotrophic culture of this group of microorganisms has been studied almost completely (Repask, 1966), fermentation technology for this microorganism with industrial aspects is still incomplete due to many difficulties i.e. explosion and the low gas usage efficiency. The recycled gas closed circuit culture (RGCCC) system which can make high gas mass transfer with recycling of substrate gas to use it up with no loss and allows operation safety for detonation and continuous operation (Takeshita, *et al*., 1993a; 1993b). According to recent progress, the autotrophic culture of *Ralstonia eutropha* gives the possibility of fermentative production of poly (hydroxybutyrate) (PHB) (Sugimoto *et al*., 1999; Tanaka and Ishizaki, 1994).

Currently the main problem that limits PHB widespread use is the cost (compared to petrochemical based polymers). The fermentation process, substrates and product recovery are the major costs (Ling *et al*., 1997). This paper deals with the bioengineering aspects of RGCCC system for industrial application to the culture of HOB as PHB producer from cheap and available gas substrate.

Ralstonia eutropha (ACM, 1296), obtained from the Australian Collection of Microorganisms is the same strain as previously reported (Khosravi-Darani *et al*., 2004a; 2004b). The medium for the refreshment of the working culture consisted of (g/l): yeast extract, 5.0; polypepton, 10.0; NaCl, 5.0; and glucose, 10.0 adjusted to pH 7.0 with NaOH. The medium for autotrophic growth, both for inoculum preparation in a shaking flask and for main culture in a jar fermentor consisted of (g/l): $(NH_4)_2SO_4$, 5.0; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.2; and $CaSO_4$, 0.004 adjusted to pH 7.0 with NaOH. Twenty mg of separately sterilized $FeSO_4.7H_2O$ was added to 1 Liter of the medium. Hydrogen gas and pure oxygen (99.9% purity) were provided from a 7 and 1.5 kl cylinders.

The flask culture for inoculum preparation was conducted in a 500 ml shaking flask with a rubber stopper in which a glass tube with a piece of silicone

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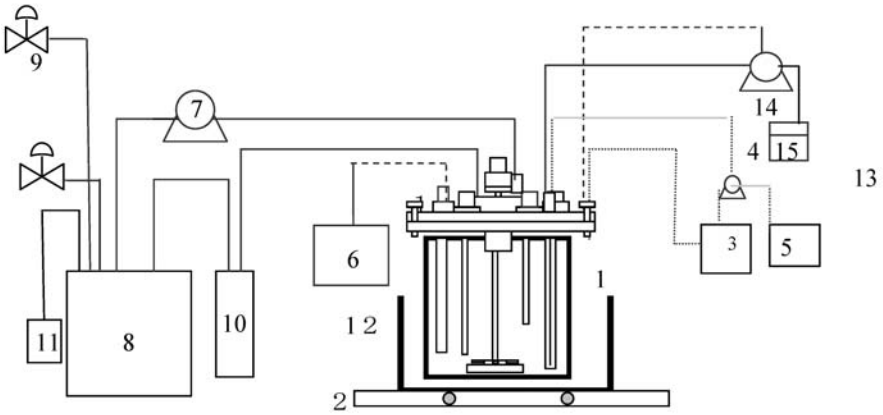

Figure 1. Schematic diagram of RGCCC system used to produce PHB by *R. eutropha* (1- jar fermentor; 2- agitation temperature controller; 3- pH controller; 4- alkali tubing pump; 5- alkaline solution; 6- DO recorder; 7- gas recycling pump; 8- gas reservoir; 9- gas sampling port; 10- trap for condensed water; 11- saline water; 12- water bath; 13- gas inlet; 14- antifoam pump; 15- anti foam reservoir).

tube sealed with a screwed sealer was inserted. The gas mixture, H_2:O_2:CO_2 = 7:2:1, prepared through a simple manifold glassware pipe work was introduced into the evacuated flask. The RGCCC system was the same as the system reported in 1994 using a mini-jar fermentor (total volume 200 ml, Able Co., Ltd., Tokyo) with instrumentation to control culture conditions (Tanaka and Ishizaki, 1994). Figure 1 shows a schematic diagram of RGCCC system used to produce PHB by *R. eutropha.*

The gas composition was estimated by reading a vacuum gauge during gas filling and finally confirmed by gas chromatography. The gas consumption during fermentation was known by the level change of saline measured from a ruler attached on the wall of the bottle. After inoculation, the cells grew by consuming of the gas which was recycled between the fermentor and gas chamber. Thus the gas as a substrate for this fermentation can be used without any loss because no exhaust gas was produced in the fermentation system. The working volume of fermentor was 100 ml. The pH was maintained at 7.0 by automatic addition of 4% ammonia. The temperature, agitation speed and gas feeding rate were kept at 30ºC, 1400 rpm and 200 ml/min (2 vvm), respectively. All stoppers and connections were carefully sealed by packing materials and bond. After the piping between the jar, bottle, and other instruments was checked, the mixed gas used for fermentation was put into the system without inoculum. Changes in gas composition were checked for possible leakage of the mixed gas.

Dissolved oxygen tension in the culture broth was measured by a membrane electrode S-Type (Able Co. Ltd., Tokyo). The protein concentration was determined by Bradford assay (Bradford, 1976). Absorbance was measured at 595 nm by spectrophotometer (Cary 50, Varian, Australia) after treatment with Coomassie brilliant blue G-250 (Sigma). Determination of PHB content in the cell was the same as reported previously (Braunegg *et al*., 1978; Khosravi-Darani *et al*., 2004a; 2004 b).

Gas consumption in terms of individual substrate gases *vs.* fermentation time is shown in Figure 2. The data plotted were restricted to the exponential growth phase (μ=cte) and the gas consumption rate (q=cte) for individual substrate gases during this phase was maintained constant. Thus the yield factors for the substrate gases remained constant for the exponential growth phase ($q=\mu/y$). Figure 3 shows the results of specific growth rate for cultures with different oxygen concentrations in the gas mixture which were conducted to examine the effects of the partial pressure of oxygen in the gas phase. Figure 4 shows a batch culture profile when mineral nutrients were supplied during the cultivation. The results shows that the cell concentration increases to about 16 g/l after 40 h of cultivation.

Batch culture was carried out using the recycled gas closed circuit culture system and a typical course of cell growth with the variation of the oxygen concentration in liquid and gas phases are shown in Figure 4. After a 8 h lag phase reaches to a constant specific growth rate. When the dissolved oxygen concentrationapproached the critical value (near zero), cell growth was repressed and followed linear growth kinetics to reach 17 g/l of cell concentration. As Figure 4 shows while the oxygen partial pressure was nearly zero, cell concentration increased from 10 to 16 g/l due to PHB accumulation in the cells.

Cultures with different oxygen concentrations in the gas mixture were conducted to examine the effects

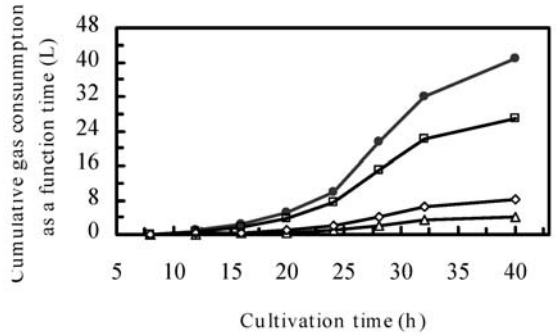

Figure 2. Relation of gas consumption increase during culture. Symbols: H_2 (□), O_2 (◊), CO_2 (△), and the gas mixture (•).

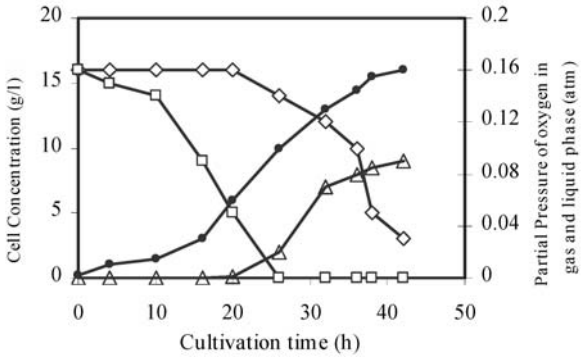

Figure 4. Autotrophic growth curve of *R. eutropha* under the gas composition H_2:O_2:CO_2 = 7:2:1 in RGCCC system. Symbols: cell concentration (•), PHB concentration (△), partial pressure of oxygen in gas phase (◊), and in liquid phase (□).

of the partial pressure of oxygen in the gas phase. The results are shown in Figure 3 and the data showed that in the range of very low oxygen concentrations, the specific growth rate increased with increasing the oxygen concentration, but after the maximum specific growth rate was attained at about 0.05 atm of oxygen partial pressure, the growth rate decreased with increases in the oxygen concentration. When the oxygen concentration was beyond 0.3 atm, the cell growth almost ceased. On the contrary the maximum cell mass concentration reached at the end of the exponential phase was increased in proportion to the increasing in the oxygen concentration. The results observed were similar to the behavior of maximum concentration of cells (Fig. 3) under oxygen limited condition was equal to reports obtained by Tanaka and Ishizaki (1994). There are a number of previous reports regarding the effect of oxygen concentration on the culture of HOB (Ishizaki *et al*., 2001; Ishizaki and Tanaka, 1990). As shown in Figure 3 the specific growth rate above the critical value of dissolved oxygen concentration depends upon the initial gas concentration and the

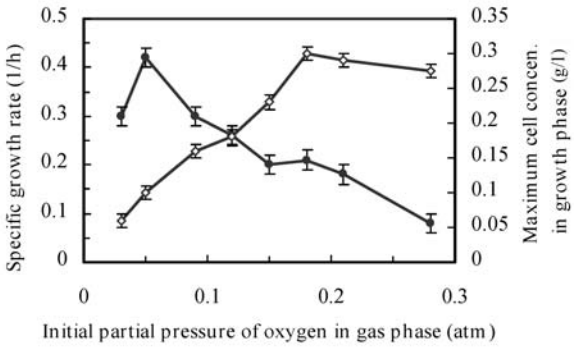

Figure 3. Effects of initial partial pressure of oxygen in the gas phase on specific growth rate (•) and maximum cell concentration (◊) in exponential growth phase.

highest specific growth rate was observed at the low initial oxygen concentration of 0.05 atm.

The biotechnological investigation on equipment and operation for fermentative PHB production using gaseous substrate for guarantee safety from explosion will be a future task. Regarding to report of Lee *et al*. (1999) low cost production of PHB and medium-chain-length PHA (ca.U.S.$ 2 kg^{-1}) can be conducted by an effective production strategy to attain a productivity greater than 4 g/l at a content of 80% of dry cell weight with a cheap carbon source. So, in PHB production from CO_2, the improvement of the polymer content in the cell and productivity is the key for the practical application. The screening of a high growth rate autotrophic bacterium, which produces high content of PHB as a primary metabolite from CO_2 are expected in the near future.

In this study, a RGCCC system for PHB production was established by a hydrogen oxidizing bacterium and it was found that in the case of *Ralstonia eutropha* (ACM, 1296) energy utilization efficiency decrease in PHB production stage due to oxygen deficiency, which agree with the results obtained by *Alca ligenes eutrophus* ATCC 17697^T (Ishizaki and Tanaka, 1994). Therefore, the application of a high growth rate microorganism, which is resistant to changes in oxygen and even carbon monoxide concentration in gas phase, will be a future task. Apparently this ability makes the bacterium suitable for consumption of exhausted gas from industrial plants. The results will be reported in the near future.

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