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CONTINUOUS TWO STEP BIOCONVERSION OF 4-OXOISOPHORONE (OIP) TO 4-HYDROXY-2,2,6-TRIMETHYLCYCLOHEXANONE (4-HTMCH) BY THERMOPHILIC BACTERIA

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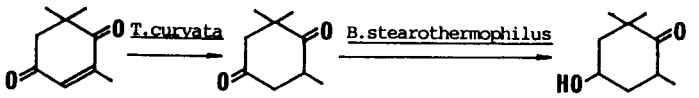
Summary

The continuous two step conversion of 4-oxoisophorone(OIP) to 4-hydroxy-2,2,6-trimethylcyclohexanone(4-HTMCH) via dihydrooxoisophorone(DOIP) was carried out using two types of thermophilic growing cells, *Thermomonospora curvata* and *Bacillus stearothermophilus*, in sequential connected continuous stirred tank reactor(CSTR) and hollow fiber reactor system. For more than 80 hours operation, 46 % of OIP was converted to 4-HTMCH with productivity of 179 mg/h/l.

Introduction

4-Hydroxy-2,2,6-trimethylcyclohexanone(4-HTMCH) is a useful intermediate in the synthesis of naturally occurring optically active carotenoids. Leuenberger et al. proposed a combination of microbial and chemical processes for the synthesis of 4-HTMCH.[3] In our previous study[7] we established a sequential two-step bioconversion process which produces 4-HTMCH from oxoisophorone(OIP) via dihydrooxoisophorone(DOIP) using two types of thermophiles, *T.curvata* and *B.stearothermophilus* in batch culture without removal or treatment of cells from the first reaction mixture. (Figure 1)

Applications of two-step bioconversions in the production of organic compounds have been investigated by several



OIP DOIP 4-HTMCH

Figure 1 : Sequential two-step bioconversion of OIP to 4-HTMCH by *Thermomonospora curvata* and *Bacillus stearothermophilus*

researchers.[1-5,9-12] However, the continuous operation of such systems is not often described.

In this paper, we investigated the continuous two-step bioconversion of OIP to 4-HTMCH via DOIP using two types of thermophiles, based on our previous study.[7] Since both microbial conversions are closely associated with cell growth, maintenance of cell growth during the conversion is essential to achieve high productivity. Here we report continuous two-step bioconversion utilizing immobilized cells in the first CSTR reactor and an air bubbling hollow fiber reactor with cell bleeding and cross flow filtration.

Materials and Methods

1. Chemicals

4-Oxoisophorone(OIP) was supplied by Japan Tobacco Inc.. Bacto tryptone (Difco) and Bacto yeast extract (Difco) were purchased from Wako Pure Chemical Industries Co., Ltd., Japan. Other chemicals were of reagent grade.

2. Microorganisms and Cultivation

Thermophilic bacteria *Thermomonospora curvata* IFO-12384 [7] and *Bacillus stearothermophilus* NK86-151 [6] isolated from soil were used in this study. Medium containing 2.0% Bacto tryptone, 1.0% Bacto yeast extract, 0.3% glucose, 1.0% glycerol and 0.3% sodium chloride in tap water (pH 7.0) was used throughout this study. Each strain was inoculated into 100 ml medium in a 500 ml flask and then incubated at 50°C on a rotating shaker for 12 hours.

3. Bioreactor System

A 300 ml spinner flask (80 mm I.D.x 175 mm, Shibata Glass Co., Ltd., Japan) with 75 ml working volume was used as the first reactor(CSTR). Air-bubbling was achieved with teflon tube submerged in medium and stirring was achieved with a magnetic stirrer. The hollow fiber reactor (Kuraray KL-F-401) was supplied by Kuraray Co., Ltd., Japan and used as the second reactor. It consisted of polyvinyl-alcohol(PVA) fibers (90% cut off size; 0.1μm, surface area; 0.1 m^2). Air-bubbling was from the bottom of a reactor. The culture in two reactors were mixed homogeneously by

stirring or air-bubbling, no dead zone and no cell settling were observed

T.curvata and *B.stearothermophilus* cells were prepared by the method descrived above. *T.curvata* cells were immobilized in poly-acrylamide(PAA) gel as was described previously[8], and were used in the first reactor(CSTR) with 75 ml of fresh medium. After 6 hours batch cultivation in two reactors, fresh medium containing OIP(3 mg/ml) was fed continuously to the first reactor and an equal volume was transferred to the second reactor. In the second reactor, equal volumes were also removed continuously with cross-flow filtration and bleeding to avoid the working volume change. The aeration rate was 2-3 vvm, and the temperature was kept at 50°C. The schematic diagram of the reactor system is shown in Figure 2.

4. Analytical Methods

Samples (1 ml) of the reaction medium were extracted with ethyl acetate (1 ml). The organic layer was separated and assayed by gas chromatograph performed on a GC-6AM gas chromatograph (Shimadzu Co., Ltd., Japan) equipped with a flame ionization detector using a stainless steel column (3 mmϕ x3 m) packed with PEG 20M 10% on Chromosorb WAW 80-100 mesh (Shimadzu Co.,

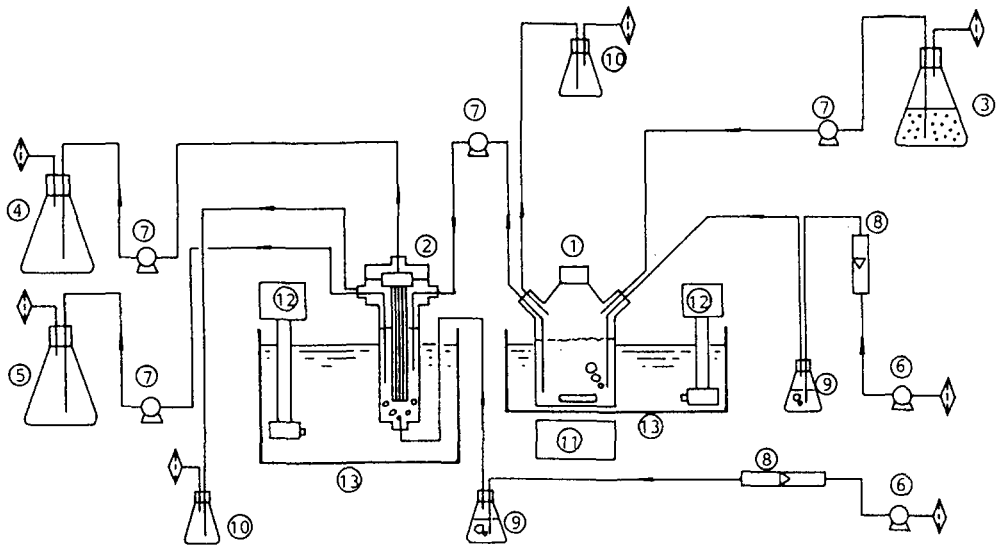


Figure 2 : Schematic diagram of combined CSTR and hollow fiber reactor system
(1)CSTR, (2)hollow fiber reactor, (3)medium reservior, (4)filtrate reservior, (5)bled culture reservior, (6)air pump, (7)peristaltic pump, (8)flow meter, (9)humidifier, (10)liquid gas separater, (11)magnetic stirrer, (12)heater, (13)water bath

Ltd., Japan) at 210°C (isothermal) with argon as the carrier gas at 80 ml/min.

Cell concentration in the culture was determined by optical density at 660nm (OD_{660}) using a Hitachi spectrophotometer Model 101 (Hitachi Co., Ltd., Japan).

Volumetric productivity is expressed as the amount of product produced for 1 hr in 1 l working volume (containing gel phase) of a reactor.

Results and Discussion

Figure 3 shows the time course of OIP conversion by immobilized *T.curvata* in the first reactor(CSTR). After 50 hours of operation, the extent of conversion reached 83%. The steady state was then observed. Then effluent from the first reactor containing 2.49 mg/ml of DOIP and 0.51 mg/ml of OIP was then started to be transferred continuously to the second (hollow fiber) reactor. Figure 4 shows the time course of bioconversion at the second reactor. In the second reactor, 90% of spent medium was cross filtered with hollow fiber membrane and remains were bled directly from shell space of hollow fiber reactor to maintain high viable cell mass. Concentration of 4-HTMCH increased gradually and reached 1.38 mg/ml, after 80 hours operation. Since by the second thermophile, *B.stearothermophilus*, OIP was also converted to DOIP, but with lower efficiency compared with the first thermophile, *T.curvata*, concentration of DOIP and OIP were 1.5 mg/ml and 0.12 mg/ml,

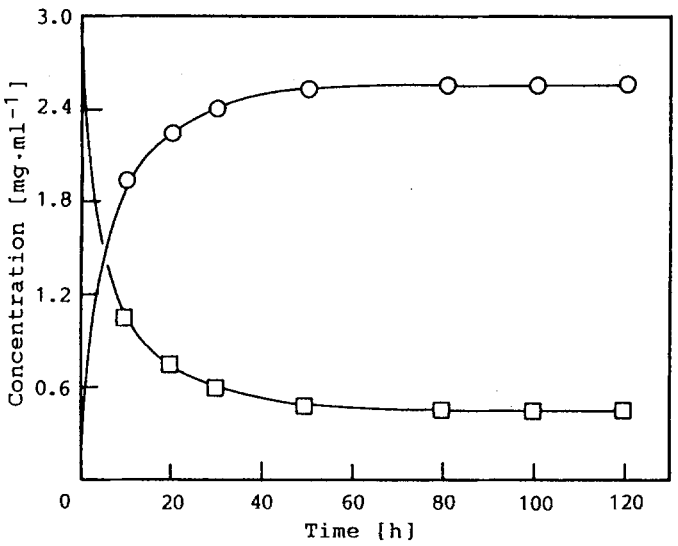


Figure 3 : Time course of OIP conversion in the first reactor(CSTR)
○ : OIP, □ : DOIP

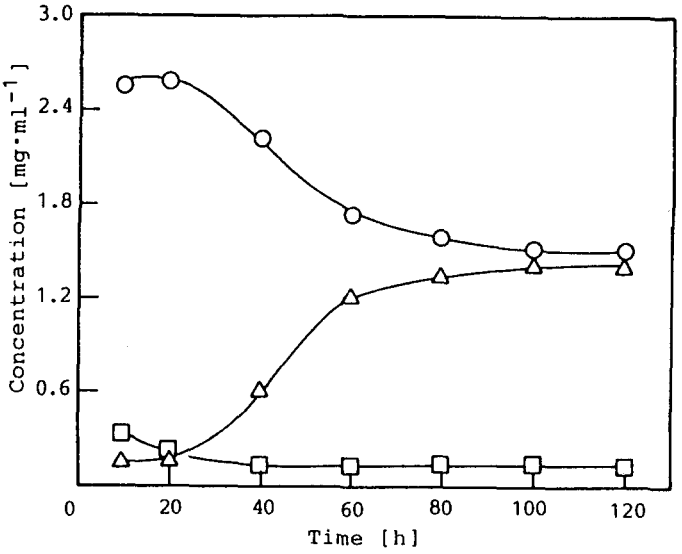


Figure 4 : Time course of OIP and DOIP conversion in the second reactor(hollow fiber)
○: OIP, □: DOIP, △: 4-HTMCH

respectively. The productivity of 4-HTMCH was 179 mg/h/l and total conversion ratio based on supplied OIP was 46%.

In a sequential two-step conversion removal or treatment of cells in the first reaction mixture is often required to achieve the second reaction. In addition, in a bioconversion associated with cell growth, cell growth and high viable cell mass are required to get high productivity. Therefore we examined continuous two-step conversion of OIP to 4-HTMCH by growing cells of thermophiles without removal or treatment of cells using consecutive bioreactors. In combination of CSTR using PAA immobilized *T.curvata* cells and hollow fiber reactor using *B.stearothermophilus* cells 46% OIP was converted continuously to 4-HTMCH via DOIP. This method can be applied to industrial use because it needs neither cell removal nor treatment of medium.

Acknowledgment

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