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Whole Cell Bioconversion of Ricinoleic Acid to 12-Ketooleic Acid by Recombinant *Corynebacterium glutamicum*-Based Biocatalyst

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The biocatalytic efficiency of recombinant *Corynebacterium glutamicum* ATCC 13032 expressing the secondary alcohol dehydrogenase of *Micrococcus luteus* NCTC2665 was studied. Recombinant *C. glutamicum* converts ricinoleic acid to a product, identified by gas chromatography/mass spectrometry as 12-ketooleic acid (12-oxo-*cis*-9-octadecenoic acid). The effects of pH, reaction temperature, and non-ionic detergent on recombinant *C. glutamiucm* whole cell bioconversion were examined. The determined optimal conditions for production of 12-ketooleic acid are pH 8.0, 35°C, and 0.05 g/l Tween80. Under these conditions, recombinant *C. glutamicum* produces 3.3 mM 12-ketooleic acid, with a 72% (mol/mol) maximum conversion yield, and 1.1 g/l/h volumetric productivity in 2 h; and 3.9 mM 12-ketooleic acid, with a 74% (mol/mol) maximum conversion yield, and 0.69 g/l/h maximum volumetric productivity in 4 h of fermentation. This study constitutes the first report of significant production of 12-ketooleic acid using a recombinant *Corynebacterium glutamicum*-based biocatalyst.

Keywords: 12-Ketooleic acid, ricinoleic acid, secondary alcohol dehydrogenase, whole cell bioconversion, *Corynebacterium glutamicum*

Introduction

There are numerous possibilities for replacing chemical techniques with biotechnological methods based on renewable resources, to meet concerns regarding environmental sustainability. One of the most widely used renewable raw materials is vegetable oils [18, 20]. Such vegetable oils and their component fatty acid provide opportunities for the manufacture of value-added products such as keto-fatty acid, which is used in the production of a variety of chemical products such as plasticizers, lubricants, detergents, cosmetics, and surfactants. A number of these compounds may be produced using bioprocesses generating lower environmental pollution than when produced by chemical processes [9].

Several reports indicate that microorganisms can convert unsaturated fatty acids to keto-fatty acids through bioprocesses. 10-Ketostearic acid is produced by *Flavobacterium* sp. DS5 [4] and *Staphylococcus warneri* [10]. 7,10-Di-hydroxy-8(E)-octadecenoic acid is produced by *Pseudomonas* sp. PR3 [5]. Strains of *Nocardia* [8] and *Flavobacterium* [3] produce 10-hydroxy-12-octadecenoic acid.

Corynebacterium glutamicum, a nonpathogenic, nonsporulating, gram-positive soil bacterium, has been widely used for biotechnological industrial applications [1], and is considered one of the most interesting microorganisms for use as a biocatalyst in cofactor-dependent reactions.

The whole cell catalyst shows several advantages over the cell-free system. Namely, the ability that the catalyst can be re-used for several bioconversion reactions without any loss of activity [13].

This study is one of the first reports examining the

biocatalytic efficiency of *C. glutamicum* in converting ricinoleic acid to 12-ketooleic acid through whole cell bioconversion. Products were identified using gas chromatography/mass spectrometry (GC/MS). The reaction conditions, including pH, reaction temperature, and non-ionic detergents, for the production of 12-ketooleic acid from ricinoleic acid by recombinant *C. glutamicum* whole cell bioconversion were optimized. Using these optimized reaction conditions, we performed whole cell bioconversion in buffer solution and through fermentation.

Materials and Methods

Microbial Strains and Culture Medium

C. glutamicum ATCC 13032 was used throughout this study for 12-ketooleic acid production. *Escherichia coli* DH5α cells (Dong-In Biotech, Korea) were used for manipulation of the expression vector (Table 1). *C. glutamicum* seed and flask cultivation was performed at 30°C in calf-derived brain–heart infusion (BHI) medium (Becton, Dickinson and company), composed of 5 g/l beef heart, 12.5 g/l calf brain, 2.5 g/l disodium hydrogen phosphate, 2 g/l D(+)-glucose, 10 g/l peptone, and 5 g/l sodium chloride. Phosphate-buffered saline (PBS) solution (pH 7.0–8.0) and 50 mM Tris-HCl (pH 8.0–9.0) buffer were used to optimize the reaction conditions. Batch cultivation and bioconversion were performed in a 2.5 L bioreactor (Kobiotech, South Korea), with a modified CGXII medium, consisting of 20 g/l NH_4SO_4, 16.68 g/l KH_2PO_4, 13.48 g/l K_2HPO_4, 0.25 g/l $MgSO_4$, 30 mg/l protocatechuic acid, 10 mg/l $FeSO_4$, 10 mg/l $MnSO_4$, 10 mg/l $CaCl_2$, 1 mg/l $ZnSO_4$, 0.2 mg/l $CuSO_4$, 20 μg/l $NiCl_2$, 0.2 mg/l biotin, 5 g/l urea, 20 g/l glucose, and 50 mg/ml kanamycin [2].

Gene Cloning

The *C. glutamicum* shuttle expression vectors pCESH36, pCESI16, and pCESL10 [21], which were the derivative of vector pCES208 [12], were used for cloning of the target gene (Table 1). The vectors contain a fully synthetic promoter capable of mediating the constitutive expression of heterologous genes in *C. glutamicum* without the addition of reagents (*e.g.*, IPTG, rhamnose). Recombinant vectors pCESH36::ADH, pCESI16::ADH, and pCESL10::ADH were constructed by transferring the ADH gene (GenBank Accession No. GQ434006.1) of *M. luteus* NCTC2665 [16], digested with *Bam*HI–*Nde*I using the In-fusion HD Cloning Kit (Clontech, USA), and designed oligonucleotides (Table 1). The constructed vector was transformed into *C. glutamicum* using the electroporation shock and heat shock method [14, 17].

Whole Cell Bioconversion in Flasks

Seed cultures were grown in 14 ml polystyrene round-bottom tube flasks containing 3 ml of BHI medium at 30°C and 200 rpm. Flask cultures were grown in 500 ml baffled flasks containing 100 ml of BHI medium inoculated with 1% (v/v) of the seed culture at 30°C and 200 rpm. Cells were harvested at the stationary growth phase by centrifugation (Supra 22k; Hanil, South Korea) at 4,500 ×*g* for 15 min at 4°C. Cells were washed twice with 50 mM Tris-HCl (pH 8.0) buffer and resuspended in the same buffer. Whole cell bioconversion was initiated in 50 mM Tris-HCl (pH 8.0) buffer containing 5.6 g/l cell dry weight at pH 8.0, 35°C, and 200 rpm.

Whole Cell Bioconversion in Bioreactors

Seed cultures were grown in 14 ml polystyrene round-bottom tube flasks containing 3 ml of BHI medium at 30°C and 200 rpm. Then 1% (v/v) of *C. glutamicum* harboring pCESL10::ADH seed

Table 1. Strains, vectors, and oligonucleotides used in this study.

Strain, vector, or oligonucleotide	Characteristics or sequence	Source or restriction site
Strains		
DH5α	F-(80d lacZ M15) (lacZYA-rgF)U1691 hsdR17	RBC
	(m+)recA1 endA1 relA1 deoR	(Real Biotech)
C. glutamicum WT	Wild-type strain ATCC 13032	KCCM
C. glutamicum pCES-L10	Wild-type strain harboring pCES-L10 (Control)	This study
C. glutamicum pCES-L10::ADH	Wild-type strain harboring pCES-L10::ADH	This study
Vectors		
pCES208	*E. coli-C. glutamicum* shuttle vector, Km^r	[12]
pCES-L10	6.7 kb, pCES208 derivative; P_{L10}, eGFP	[21]
pCES-L10::ADH	pCES-L10 containing ADH	This study
Oligonucleotides		
ADH_F	5′- GAGATAGATTGGATCCATGTCCGAGTTCACCCGTTTC-3′	*Bam*HI
ADH_R	5′- TCATGCTGTTTCATATGTCAGCCGAGCGGGGTGTC-3′	*Nde*I

culture was cultivated in 100 ml of BHI medium in a 500 ml baffle flask at 30°C and 200 rpm. For preparation of the batch culture, the flask culture was inoculated into a 2.5 L bioreactor (Kobiotech, South Korea) at 10% (v/v) inoculation volume and 1 L operating volume. Dissolved oxygen was provided by injection of filtered air at a flow rate of 1.5 vvm, and the agitation speed was maintained at 200 rpm. The culture pH was maintained at 7.0 through automatic addition of 5 M NaOH and 5 M HCl solutions until cells reached the stationary growth phase. Bioconversion was initiated subsequently by shifting the pH to 8.0 and temperature to 35°C.

Product Analysis by Gas Chromatography/Mass Spectrometry

The concentrations of the remaining fatty acids and accumulated keto-fatty acid in the reaction medium (*e.g.*, ricinoleic acid, 12-ketooleic acid) were determined as previously described [7]. The reaction medium was mixed with ethyl acetate containing 0.1 or 0.5 g/l palmitic acid as an internal standard. The organic phase was harvested after vigorous vortexing and then subjected to derivatization with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (TMS)/pyridine (1:3 (v/v)). The TMS derivatives were analyzed using a 5975 series MSD and Agilent 7890A gas chromatograph. The derivatives were separated on a nonpolar capillary column (30 m length, 0.25 μm film thickness; HP-5MS; Agilent Technologies, Palo Alto, CA, USA). A linear temperature gradient was programmed as 90°C, 5°C/min to 280°C, with an injection port temperature of 230°C. Samples were injected at a split ratio of 1:5. Mass spectra were obtained by electron impact ionization at 70 eV. Scan spectra were obtained within the 100–600 *m/z* range. Selected ion monitoring was used for the detection and fragmentation analysis of the reaction products [6].

Results and Discussion

Selection of Recombinant *Corynebacterium* Strains by Whole Cell Bioconversion

For optimal yield and productivity, it is important to select appropriate promoters for the target gene. From an economic standpoint, constitutive promoters are superior to inducible promoters [11, 18]. Therefore, we used pCES208 vectors, each containing a synthetic promoter (H36, I16, or L10) allowing for the constitutive expression of heterologous genes, for the transformation of *C. glutamicum*.

C. glutamicum-pCESL10::ADH (*C. glutamicum* expressing

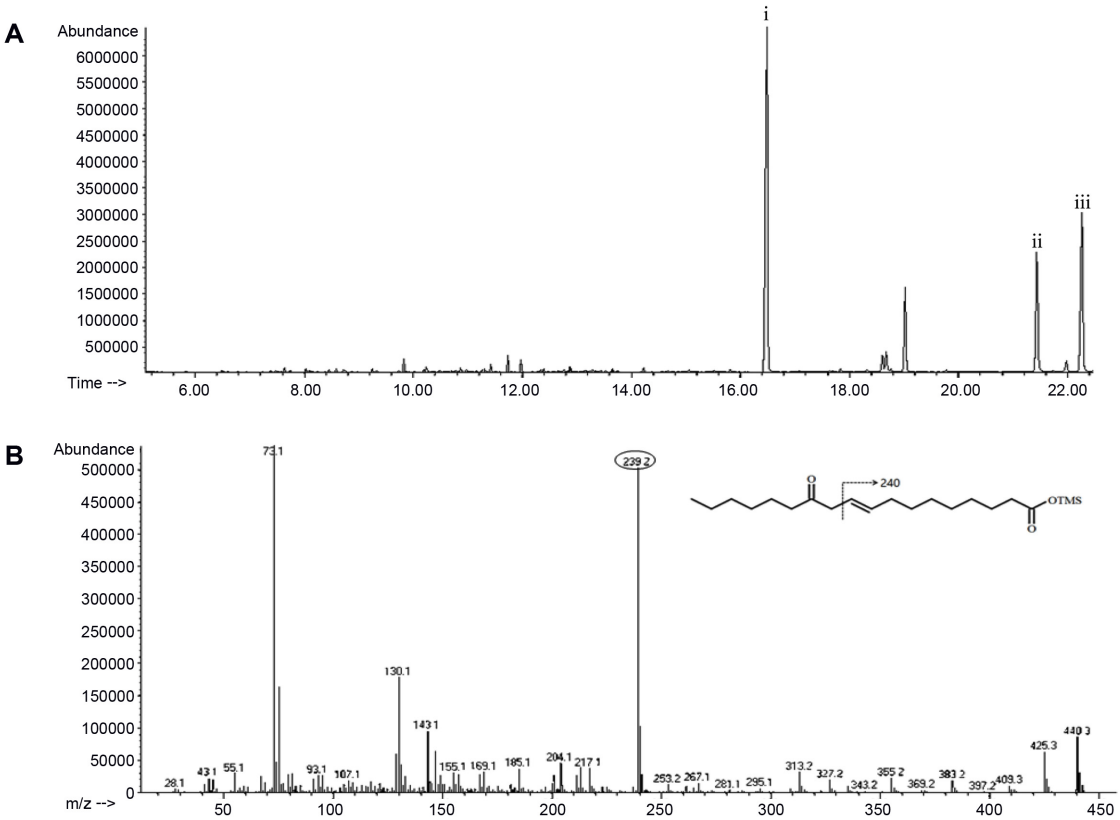

Fig. 1. Gas chromatography/mass spectrometry (GC/MS) analyses of biocenversion products.

(**A**) Gas chromatogram of trimethylsilyl (TMS) derivatives recovered after bioconversion of ricinoleic acid by recombinant *C. glutamicum*. Peaks were identified as (i) trimethylsilyl palmitate (internal standard), (ii) trimethylsilyl ricinoleic acid, and (iii) trimethylsilyl 12-ketooleic acid. (**B**) Chemical structure and GC/MS spectra of trimethylsilylated 12-ketooleic acid.

ADH under the control of the constitutive L10 promoter) produced the highest 12-ketooleic acid yield (data not shown) and therefore was selected for 12-ketooleic acid production by whole cell bioconversion.

Gas chromatographic analysis of the conversion product revealed a single, new GC peak that was determined to be 12-ketooleic acid (Figs. 1A and 1B).

Effects of pH and Reaction Temperature on the Production of 12-Ketooleic Acid by Whole Cell Bioconversion

The conversion of ricinoleic acid to 12-ketooleic acid by whole cells of recombinant *C. glutamicum* was examined by varying the pH from 7.0 to 9.0, and the reaction temperature from 25°C to 45°C, using a constant cell dry weight of 5.6 g/l. To optimize whole cell bioconversion reaction conditions, we examined the effects of pH and reaction temperature on bioconversion containing two types of buffer solutions with the addition of 5 mM ricinoleic acid and 0.05 g/l Tween 80. The optimal pH yielding the highest conversion efficiency of ricinoleic acid to 12-ketooleic acid was determined using either a 50 mM PBS buffer or 50 mM Tris-HCl buffer (Fig. 2A). The pH 8.0 conversion data indicate that the Tris-HCl buffer is more suitable than the PBS buffer for the conversion of ricinoleic acid to 12-ketooleic acid by recombinant *C. glutamicum*. The conversion efficiency of ricinoleic acid to 12-ketooleic acid using the 50 mM Tris-HCl (pH 8.0) buffer was relatively high; the relative conversion efficiency decreased at a pH higher or lower than 8.0. Therefore, pH 8.0 was determined as optimal for the conversion of ricinoleic acid to 12-ketooleic acid. In addition, we studied the effect of reaction temperature (25–45°C) on whole cell bioconversion in 50 mM Tris-HCl (pH 8.0) buffer (Fig. 2B). The conversion efficiency of ricinoleic acid to 12-ketooleic acid was poor at 40°C and 45°C compared with that at 25°C, 30°C, and 35°C. At temperatures higher than 40°C, recombinant *C. glutamicum* exhibited both decreased growth and low conversion efficiency. When the temperature range of 25°C to 35°C was examined, maximum conversion efficiency was apparent at 35°C. These results indicate that the conversion of ricinoleic acid to 12-ketooleic acid is most affected by pH and reaction temperature. Thus, optimizing the pH and reaction temperature is critical for the production of 12-ketooleic acid by whole cell bioconversion in recombinant *C. glutamicum*.

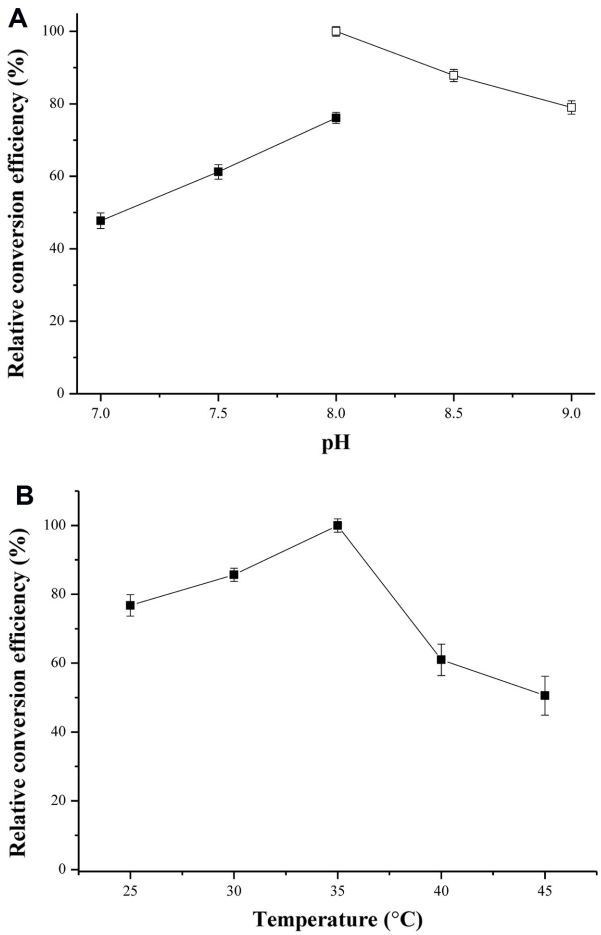

Fig. 2. Effects of pH and reaction temperature on whole cell bioconversion in recombinant *C. glutamicum*.
(**A**) Effect of pH; Whole cell bioconversion was performed in 50 mM PBS buffer (□) for pH 7.0 to 8.0, and 50 mM Tris-HCl buffer (■) for pH 8.0 to 9.0, in addition to 5 mM ricinooleic acid and 0.05 g/l Tween 80 for 2 h. (**B**) Effect of temperature; Whole cell bioconversion was performed in 50 mM Tris-HCl (pH 8.0) buffer containing 5 mM ricinoleic acid and 0.05 g/l Tween 80 for 2 h with a reaction temperature range of 25°C to 45°C.

Effects of Non-Ionic Detergents on the Production of 12-Ketooleic Acid by Whole Cell Bioconversion

Detergents are generally used to disrupt biological membranes and solubilize membrane proteins or to ensure homogeneity of soluble proteins in suspensions. Ionic detergents denature protein, whereas non-ionic detergents do not. Therefore, appropriate detergents must be chosen to avoid the denaturation and inactivation of proteins. Non-ionic detergents contribute to the uniform distribution of unsaturated fatty acids in aqueous solutions, enabling their effective utilization as substrates by microorganisms [15, 19].

Therefore, we examined which non-ionic detergents contributed the most to the production of 12-ketooleic acid from ricinoleic acid by whole cell bioconversion in

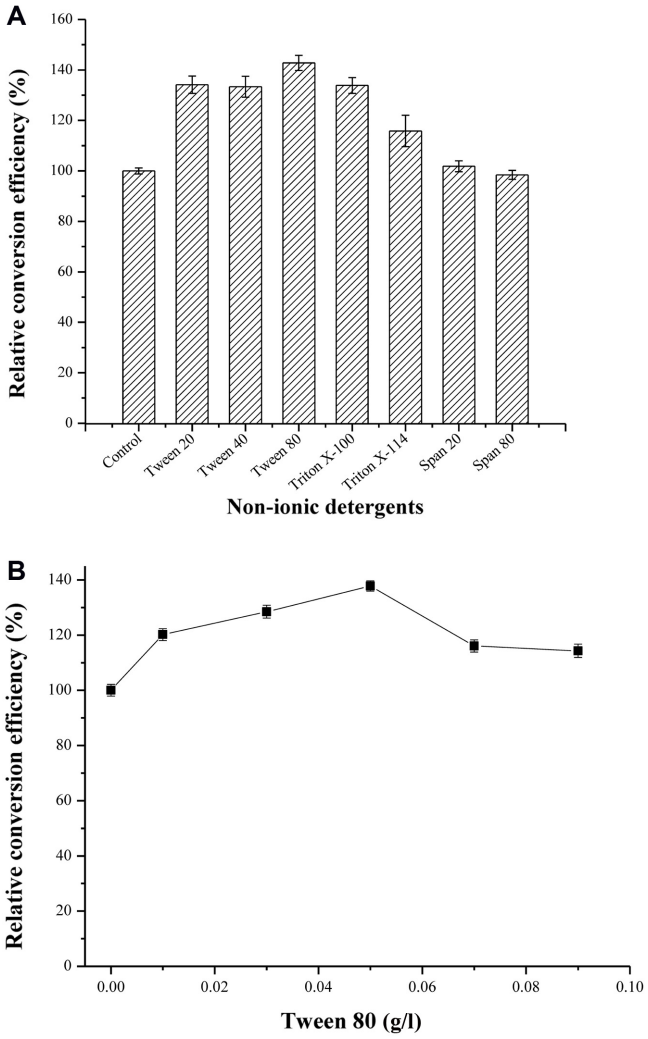

Fig. 3. Effects of detergents on the conversion of ricinoleic acid to 12-ketooleic acid by whole cell bioconversion in recombinant *C. glutamicum*.

(**A**) The effect of various types of non-ionic detergents. Whole cell bioconversion was performed in 50 mM Tris-HCl (pH 8.0) buffer containing a cell dry weight of 5.6 g/l, 5 mM ricinoleic acid, and 0.05 g/l detergent at 35°C for 2 h. (**B**) The effect of Tween 80 concentration. Whole cell bioconversions were performed in 50 mM Tris-HCl (pH 8.0) buffer containing a cell dry weight of 5.6 g/l, 5 mM ricinoleic acid, and varying concentrations of Tween 80 at 35°C for 2 h.

recombinant *C. glutamicum* (Figs. 3A and 3B). Fig. 3A demonstrates the relative conversion efficiency of 12-ketooleic acid using the non-ionic detergents Tween 20, Tween 40, Tween 80, Triton X-100, Triton X-114, Span 20, and Span 80 (at a concentration of 0.05 g/l). Of the non-ionic detergents examined, Tween 80 resulted in the highest production of 12-ketooleic acid; a 1.4× increase compared

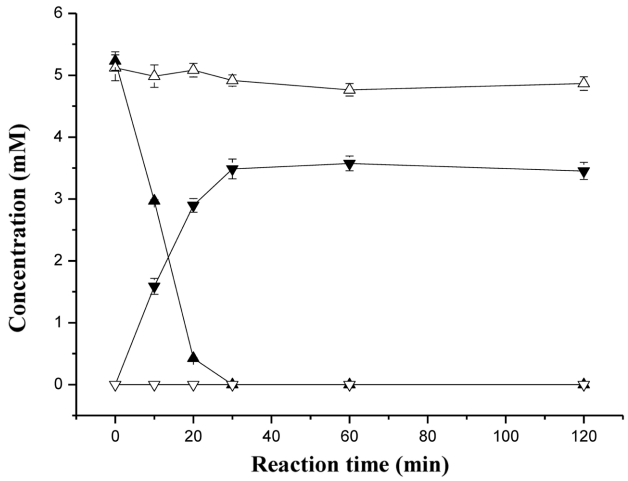

Fig. 4. Whole cell bioconversion of ricinoleic acid to 12-ketooleic acid under optimized conditions in 50 mM Tris-HCl (pH 8.0) buffer containing 5 mM ricinoleic acid and 0.05 g/l Tween 80 for 2 h.

Subsequent to cultivation in BHI medium, cells were harvested at the stationary growth phase and resuspended in reaction medium to a cell density of 5.6 g/l cell dry weight. Symbols indicate concentration of ricinoleic acid (△), and 12-ketooleic acid (▽) for *C. glutamicum*-pCESL10; and of ricinoleic acid (▲) and 12-ketooleic acid (▼) for *C. glutamicum*-pCESL10::ADH.

with the control sample (no added non-ionic detergents). These results indicate that non-ionic detergents are required to improve the ability of recombinant *C. glutamicum* to produce 12-ketooleic acid from ricinoleic acid. The conversion efficiency of 12-ketooleic acid relative to the concentration of Tween 80 (0–0.09 g/l) used is shown in Fig. 3B. Addition of Tween 80, at any concentration, improved the conversion efficiency; the highest production of 12-ketooleic acid was attained at a concentration of 0.05 g/l.

Whole Cell Bioconversion Based on Buffer Solution Under the Optimized Conditions

Three milliliters of recombinant *C. glutamicum* seed culture was grown in a 14 ml round-bottom tube overnight; flask cultivation was performed in BHI medium containing 50 mg kanamycin/ml at 30°C and 200 rpm until reaching the stationary phase. Subsequent to cell harvesting and resuspension, whole cell bioconversion of ricinoleic acid to 12-ketooleic acid was performed under the optimized conditions in 50 mM Tris-HCl (pH 8.0) buffer containing 5 mM ricinoleic acid and 0.05 g/l Tween 80 for 2 h. (Fig. 4). The resulting data, analyzed using GC/MS, indicate a maximum conversion yield of 72% and maximum volumetric productivity of 1.2 g/l/h.

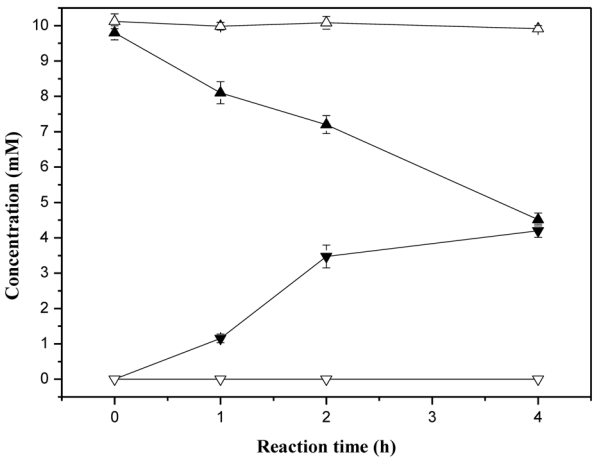

Fig. 5. Whole cell bioconversion of ricinoleic acid to 12-ketooleic acid in CGXII medium under the optimized conditions. At stationary growth phase, 10 mM ricinoleic acid and 0.05 g/l Tween 80 were added and the reaction was performed for 4 h. Symbols indicate concentration of ricinoleic acid (△), and 12-ketooleic acid (▽) for *C. glutamicum*-pCESL10; and of ricinoleic acid (▲) and 12-ketooleic acid (▼) for *C. glutamicum*-pCESL10::ADH.

Whole Cell Bioconversion Depends on the Medium Under Optimized Conditions

For industrial-scale production, performing bioconversion reactions directly in the medium is simpler and cheaper than using a buffer-based system. Therefore, whole cell bioconversion of ricinoleic acid to 12-ketooleic acid was performed in modified CGXII medium under the optimized reaction conditions (pH 8.0, 35°C) in a 2.5 L bioreactor (Kobiotech, South Korea). When cells reached the stationary growth phase, bioconversion was initiated by the addition of 10 mM ricinoleic acid and 0.05 g/l Tween 80 (Fig. 5). GC/MS analysis revealed that 5.3 mM ricinoleic acid was consumed, and 3.9 mM 12-ketooleic acid was produced. The maximum volumetric productivity was 0.69 g/l/h and the maximum conversion yield was 74% (mol/mol). The result indicated that the production of 12-ketooleic acid by whole cells of recombinant *C. glutamicum* under optimized conditions would be a good approach in terms of conversion yield; however, the productivity was lower than that of buffer-based whole cell bioconversion. These results will be beneficial for the biological production of 12-ketooleic acid at an industrial scale.

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