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TITLE: Production of CP4 EPSP Synthase in a 100 Liter Recombinant *Escherichia coli* Fermentation

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ABSTRACT: This report documents the fermentation process used to produce purified CP4 EPSP Synthase needed for safety assessment studies in support of EPA regulated registration of transgenic plants. CP4 EPSP Synthase was produced by recombinant *E. coli* in a single 100 liter fermentation. Complex medium and a glucose fed-batch fermentation process were used for production. The production organism was *E. coli* GB100 harboring the plasmid pMON21104. Expression of CP4 EPSP synthase from pMON21104 was driven by the recA promoter.

KEYWORDS: Fermentation process, Safety assessment, CP4 EPSP Synthase

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PRODUCTION OF CP4 EPSP SYNTHASE IN A 100 LITER RECOMBINANT *ESCHERICHIA COLI* FERMENTATION
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INTRODUCTION

The potential to commercialize Roundup™ tolerant soybeans was made feasible by creating transgenic soybean plants containing the CP4 EPSP synthase gene. As a result, purified CP4 EPSP synthase was required for safety assessment studies. Purification of sufficient quantities of CP4 EPSP synthase from plants would be difficult to achieve, so recombinant *E. coli* was used as an alternative production system to generate the desired quantities of protein needed for safety assessment testing. The CP4 EPSP synthase was produced in the *E. coli* cytoplasm as a soluble product. A collaborative effort between the New Products Division of MAC and the Bioprocess Technology group of MCR succeeded in producing sufficient quantities of CP4 EPSP synthase for protein characterization and safety assessment testing.

MATERIALS AND METHODS

Culture

The host/vector system used to produce CP4 EPSP synthase was *E. coli* GB100 harboring plasmid pMON21104. Plasmid pMON21104 was approximately 4.61 Kb in size and contained the *E. coli* structural gene for CP4 EPSP synthase. Upstream of the structural gene was the Gene 10 leader ribosome binding site from phage T7 and the recA promoter which drives expression of CP4 EPSP synthase. pMON21104 also contains the pBR327 origin of replication, the T7 transcription terminator from phage T7 and the spectinomycin resistance gene as a selectable marker (1).

The standard seed culture inoculum consisted of 1.0 ml aliquots of late-logarithmic culture grown in Luria broth (Difco) containing 75 ppm spectinomycin. The culture was concentrated and resuspended in fresh Luria broth containing 10% glycerol (v/v). The seeds were stored in vapor phase liquid nitrogen at or below -100° C. This culture is retained in the BPcT culture collection as MS#540 and the seed vial lot is VL#394 (2).

Fermentation Process

Primary Seed Culture

2.8 liter Fernbach flasks containing 1.0 liter of M9 minimal salts medium supplemented with 10 g/l casamino acids, 75 ppm spectinomycin and 0.5 ml of UCON LB 625 polyalkylene glycol antifoam were used as primary seed cultures (Table 1). The flasks were capped with Bellco silicone sponge flask closures and autoclaved for 30 minutes at 121°C and 15 psi pressure. Each of six flasks was inoculated with a 1.0 ml seed vial and allowed to grow for 8 hours at 37°C by shaking on a New Brunswick G-53 air shaker set at 250 rpm. The A_{550} of a

dummy flask, which was not carried to the next stage, was 2.0 when the five flasks were transferred to the 100 liter production fermentor.

Production Fermentation - Run # 307-92019

The production fermentation to produce CP4 EPSP synthase was carried out in a 150 liter Chemap fermentor (BPcT Tank #307) which was charged with approximately 100 liters of M9 medium supplemented with 10.0 g/l casamino acids (Table 1). The fermentor was sterilized for 30 minutes at 121°C and 15 psi pressure.

Control parameters were:

Airflow	150 LPM
pH	7.0 with 29% NH_4OH or 20% H_3PO_4
Agitation	500 RPM
Pressure	10 PSI
Temperature	37.0°C
Mass (final)	105 KG
Dissolved O_2	Maintained > 30% saturation by the following successive steps: A. increasing pressure to 15 PSI. B. increasing agitation in 100 RPM increments to a maximum of 600 RPM.
Glucose	Initial charge 10.0 g/l. Feed a 50% (wt/vol) glucose solution to control at 2-5 g/l.
Induction	Dose nalidixic acid to a final concentration of 50 ppm at O.D. 550 nm = 12.65.

Off-gas analysis of this fermentation was performed and the volumetric oxygen uptake rate (OUR), carbon dioxide evoution rate (CER) and respiratory quotient (RQ) are shown in Figure 1. Operational details relating to the seed and production fermentations can be found in the batch record (3).

At four hours post-induction the fermentor was chilled to 15°C before transfer to a chilled holding tank. Chilling was maintained until the culture was centrifuged in a Sharples centrifuge. Approximately 105 kg of broth was centrifuged to recover 6.373 kg of cell paste. The final volumetric yield of cell paste was 59.5 g/l. One-half of the cell paste was frozen at -25°C. The remaining half of the paste was lysed by homogenization and the soluble fraction was precipitated by ammonium sulfate. The isolation run number was #I92039 (4).

Analytical

Microbial Contamination Testing

The vial lot #394 was tested for a variety of types of microbial contaminants. Phenylethanol agar (Difco) was used to screen for the presence of gram positive contaminants, *Pseudomonas* isolation agar (Difco) was used to select for *Pseudomonas* contamination, Mycophil agar with low pH (BBL) was used for detection of fungi and yeast and tryptic soy agar was used to test for other types of contaminants not culturable on selective and differential media. Moderate inocula from two vials (representing 2.5% of the preparation) were plated onto contamination detection media and incubated appropriately for the types of organism to be detected (5).

The fermentor was sampled before inoculation and at harvest for microbial contamination testing.

The preinoculation media samples from the fermentor were tested for sterility by membrane filtration of 1.0 ml samples through 0.45μ pore filters and incubating filters on tryptic soy agar at 35°C for 72-96 hours (6).

The preinoculation and harvest fermentation samples were collected, refrigerated and tested for contamination with a method similar to that for the seed preparation except that the medium for detection of fungal contaminants was omitted (7).

Fermentation Process Monitoring and Control

Monitoring of pH, dissolved oxygen, agitation, back pressure and offgas composition was handled by standard instrumentation. Glucose concentration was monitored using a YSI glucose analyzer (8). Additionally, fermentation samples were collected at specific intervals for optical density (A_{550}) measurement (9) and pH verification.

RESULTS

Contamination Testing

Microbial contamination was not detected in the seed preparation nor in any medium or fermentation sample.

Production Fermentation

Operation and Control

Figure 2. shows the optical density and glucose concentration profiles during the run. Glucose feeding was started at hour 5.5 (thirty minutes post-induction) at a flow rate of 6.7 ml/min. The flow rate of glucose solution into the fermentor was adjusted between 10 and 15 ml/min to maintain the residual glucose concentration of 2-5 g/l during the induction phase. When the dissolved oxygen concentration dropped to 67% saturation at induction, the prescribed steps were taken to maintain a dissolved concentration >30%. The vessel pressure was increased from 10 psi to 15 psi and the agitation was increased from 500 to 600 rpm. The dissolved oxygen concentration remained above 57% saturation for the remainder of the fermentation (Figure. 3). No additional antifoam was required for the fermentation. pH was controlled at 7.0 throughout the run. Temperature remained constant at 37°C throughout the run. The culture reached an O.D. 550 nm of 12.7 at 5.0 hours post-inoculation and was induced with a final nalidixic acid concentration of 50 ppm. A final broth mass of 107 Kg was harvested at six hours post-induction. The total fermentation time was 11.0 hours. A plot of the on-line measured variables is shown in Figure 3.

CP4 EPSP Synthase Activity

Following homogenization and ammonium sulfate precipitation, the precipitate was redissolved and CP4 EPSP synthase activity in the crude was demonstrated by enzyme assay (4).

DISCUSSION

The fermentation protocol used for the one hundred liter production run was developed by duplicating the successful ten liter fermentation protocol. There were no deviations from the parameter ranges outlined in the batch record. Scale up of a seed inoculum was identical to that for the ten liter fermentations, and the performance of the culture in the shake flasks was exactly as predicted, reaching a density of 2.0 at transfer. Five mid-log shake flask cultures were used to inoculate the one hundred liter fermentor. The average specific growth rate of the culture during mid-logarithmic growth in the fermentor prior to induction was approximately 0.82 h^{-1} . The optical density profile of the fermentation, shown in Figure 2, illustrates a typical growth profile for *E. coli* in M9 + casamino acids medium. Glucose was added to the fermentor prior to inoculation at a final concentration of 13 g/l. At 0.5 hours post induction the glucose concentration dropped to 4.8 g/l and glucose feeding was initiated. The glucose concentration was controlled between 3.30 and 4.2 g/l for the duration of the fermentation (Figure 2). Following induction the minimum dissolved oxygen concentration of the culture was 47% saturation. As a result the parameters of agitation and airflow remained constant throughout the fermentation run (Figure 3).

ACKNOWLEDGEMENTS

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Table 1.

Composition of M9 Casamino Acids Medium

Component	Sterilization*	Amount/L
$Na_2HPO_4 \cdot 7H_2O$	BST	13.2 g
KH_2PO_4	BST	3.0 g
NaCl	BST	0.5 g
NH_4Cl	BST	1.0 g
Casamino acids	BST	10.0 g
Glucose 50% (wt/vol)	HST	10.0 g plus feed
$CaCl_2 \cdot 2H_2O$	HST	4.0 mg
$MgSO_4$	HST	0.3 g
Trace Metals IV 100%	FST	0.1 ml
Thiamine HCl	FST	2.5 mg
Pyridoxal phosphate	FST	25.0 mg
UCON LB 625	BST	0.5 ml

* BST = Component added to fermentor and sterilized with other similarly coded items.
HST = Heat sterilized separately from BST components.
FST = Filter sterilized and added to sterilized fermentation medium after cooling.

Composition of Trace Metals IV 100% Stock Solution

Component	Amount/Liter
H_2SO_4 (conc.)	17.0 ml
$FeCl_3 \cdot 6H_2O$	189.0 g
$ZnSO_4 \cdot 7H_20$	2.00 g
$CoCl_2 \cdot 6H_2O$	3.50 g
$Na_2MoO_4 \cdot 2H_2O$	3.50 g
$CuSO_4 \cdot 5H_2O$	4.00 g
H_3BO_3	1.00 g
$MnSO_4 \cdot H_2O$	2.50 g

Trace metals dissolved in 17 mls sulfuric acid
Q.S. to 500 mls with distilled water.
Trace metals stock solution was filter sterilized prior to use.

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Figure 1. CP4 EPSP SYNTHASE - 100 LITER GMP FERMENTATION - BATCH RECORD #307-92019
Volumetric CO2 and O2 rates and RQ

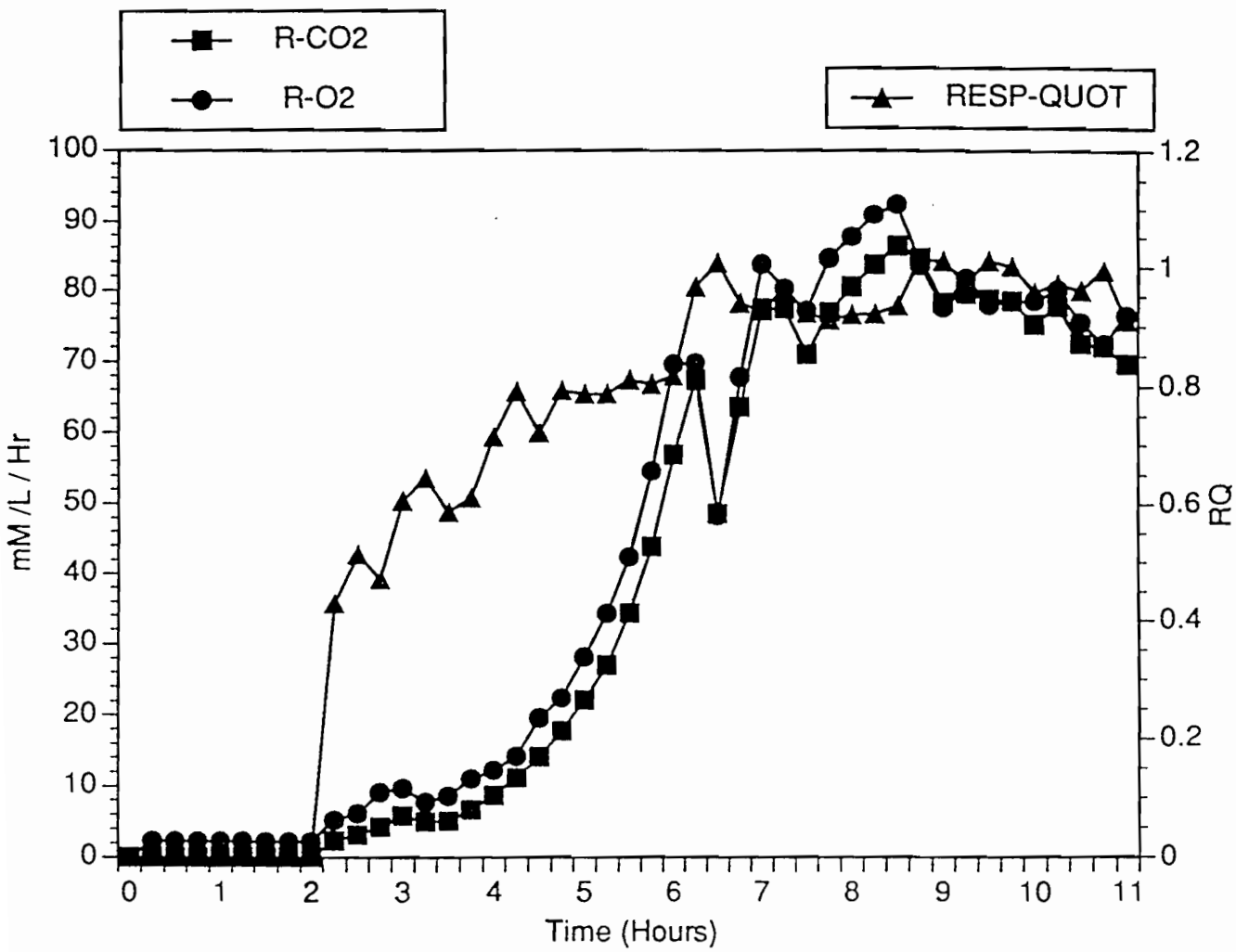

Figure 2. CP4 EPSP SYNTHASE - 100 LITER GMP FERMENTATION - BATCH RECORD #307-92019
Optical Density and Glucose Profiles

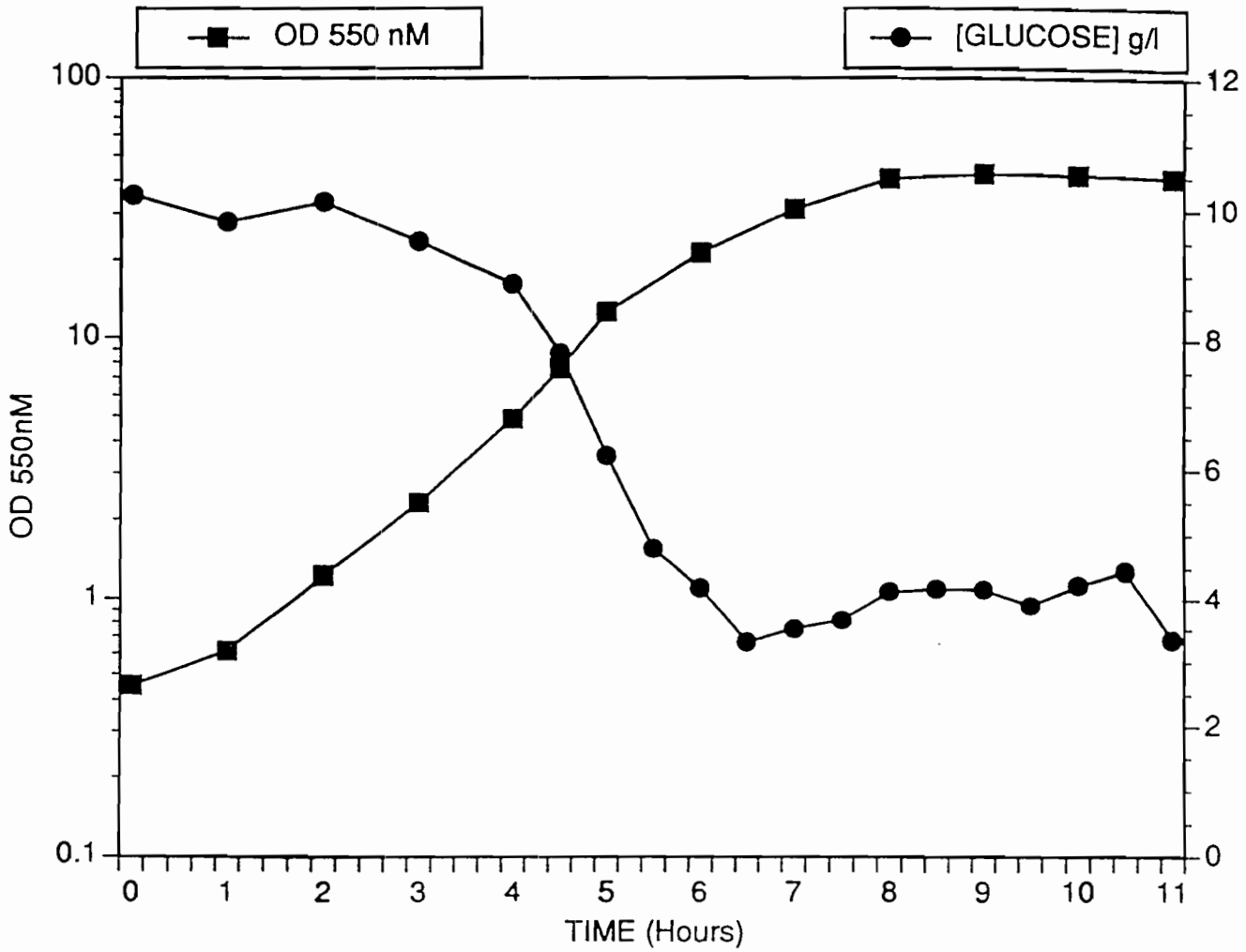

Figure 3. CP4 EPSP SYNTHASE - 100 LITER FERMENTATION - BATCH RECORD #307-92019
Run Parameter Profiles