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Enantioselectivity of sulcatone reduction by some anaerobic bacteria

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Prominent among several factors that have been reported to affect the enantioselectivity of ketone reduction by washed suspensions of microorganisms are (i) the phase of batch culture at which the organisms are harvested, and (ii) the concentration at which the ketone is supplied. Sulcatone (6-methylhept-5-en-2-one) is only poorly soluble in aqueous media, but in the present study a novel microbe friendly *organic solvent mixture was employed to present this ketone in a range of concentrations to cell suspensions of four bacteria that were then incubated in an atmosphere of hydrogen for 6 h at 30°C. The bacteria employed were* Clostridium pasteurianum, Clostridium tyrobutyricum *(two strains), and* Lactobacillus brevis *that had been harvested at various times from anaerobically growing batch cultures.* L. brevis *formed* R(−)*-sulcatol in high enantiomeric excess irrespective of its period of pregrowth in batch culture and the substrate sulcatone concentration (over the range 0.02–1.5 mM); however, in the case of all three clostridia, the enantiomeric selectivity of sulcatone reduction was substantially affected by both of these factors. It is likely that this reflects the possession by these clostridia of multiple ketone reductases differing in their* K_m *values for sulcatone and present in the organisms in differing proportions during the course of their batch culture growth.* © *1997 Elsevier Science Inc.*

Keywords: Bioreduction; enantiomeric selectivity; clostridia; *Lactobacillus brevis*; sulcatone

Introduction

Washed suspensions of microorganisms or oxidoreductase enzymes of microbial origin represent a rich source of catalysts exploitable for the bioreductive synthesis of enantiomerically pure chiral compounds from prochiral precursors.[1] Whole cell suspensions are often the preferred agents of bioreductions, since metabolically active organisms continually furnish their endogenous reductase enzyme(s) with the requisite electron donor(s) whereas a purified or partially purified enzyme needs to be coupled to an efficient (and potentially expensive) means of regeneration of the appropriate reduced cofactor.[2] The stereochemical outcome of whole cell reductions of carbonyl compounds is ultimately reliant on the activities of the enzymes possessed by the organism that participate in the reactions.[3] When only a single oxidoreductase is implicated, poor enantiotopic face specificity could account for low enantioselectivity of the biocatalyst, but low enantiomeric selectivity has more often been attributed to plural and competing oxidoreductases generating enantiomers of opposite configuration.[4] In too few studies has it been recognized that the number and relative proportions of functioning oxidoreductases may vary in the course of batch culture growth and thus, in turn, greatly influence the enantioselectivity of a bioreduction undertaken by suspensions of the harvested organisms. In such situations, various strategies may be employed to enhance, and/or direct, the stereoselectivity of the bioreduction including: growth of the microorganism in culture conditions that favor the desired activity;[5] selective inhibition of one or more of the competing enzymes;[6] and choice and maintenance of the optimal substrate concentration.[7]

When a carbonyl substrate is relatively water insoluble, the opportunity to present it to the biocatalyst at the most desirable concentration is very limited even when that substrate is first dissolved at a relatively high concentration in a (water immiscible) organic phase. It was thought that a suitable miscible organic solvent might be found which, when added to the medium, would dissolve such substrates; however, it was found that the requisite concentration of such a solvent was so high that it was often inhibitory and/or toxic to the microbial catalyst; thus, relatively water-insoluble organic solvents were studied when it emerged that a mixture of ethyl acetate with ethanol and some Tween 80 could be devised such that it was dispersed evenly throughout the medium and did not adversely affect the

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viability of suspended microbes (both yeast and bacteria).[8] The availability of this *microbe friendly* solvent mixture has enabled us to overcome the constraint posed by the low water solubility of sulcatone. The chief purpose of the present paper is to report that the stereochemistry of reduction of sulcatone (6-methylhept-5-en-2-one) by washed suspensions of clostridia (though not of *Lactobacillus brevis*) was indeed influenced by the concentration at which the sulcatone was supplied as well as by the stage during batch culture growth at which the organisms had been harvested.

Materials and methods

Bacteria

Clostridium pasteurianum ATCC 6013 and *Clostridium tyrobutyricum* strains AuxP and PON5[9] were maintained at room temperature as sporulated cultures on slants of Reinforced Clostridial Agar (LabM). *L. brevis* NCIMB 947 was maintained at 4°C as stab cultures on agar-solidified MRS medium[10] containing 2% (w/v) glucose.

Growth of bacteria

The clostridia were grown anaerobically at 35°C as batch cultures (2-l) in a defined minimal medium[9] containing either glucose 2% (w/v) or crotonate 1% (w/v) as the carbon and energy source. *L. brevis* was also grown anaerobically in 2-l batch culture but at 30°C in MRS medium with 2% (w/v) glucose. Culture growth was followed by measurement of optical density at 680 nm using a Pye-Unicam SP 1800 spectrophotometer (Pye-Unicam, Cambridge, UK).

Bioreduction with washed suspensions of bacteria

Organisms harvested by centrifugation were washed and resuspended (to about 60 mg dry weight ml^{-1}) in 0.1 M KH_2PO_4 adjusted to pH 6.9 with 4 M HCl. A quantity of this suspension (4.9 ml of the clostridia but 4.5 ml of the lactobacillus) was placed under H_2 in a 25 ml glass pressure tube sealed with a butyl rubber cap (Bellco, Vineland, NJ). Sulcatone was injected via this cap in 50 μl of a solvent mixture containing ethyl acetate:Tween 80:ethanol, 43.5:0.5:56 (v/v). Further additions were made of (a) 0.1 ml of 20 mM methyl viologen to the clostridial suspensions, or (b) 0.5 ml of glucose 2% (w/v) to suspensions of the lactobacillus. Each tube and its contents was thereafter held at 30°C for 6 h on a horizontal reciprocal shaker with a displacement of 12 cm and operating at 70 oscillations min^{-1}. At timed intervals, samples (0.1 ml) were withdrawn and the residual concentration of sulcatone determined; further additions of sulcatone (in 50 μl) of solvent mixture were made to sustain the required substrate concentration.

Determination of solvent toxicity

The toxicity to the various bacterial suspensions of the solvent mixture (with or without dissolved sulcatone) was assessed by dielectric spectroscopy[11-12] using a Biomass Monitor (Aber Instruments, Aberystwyth, UK). The measuring electrode (biomass probe) was inserted laterally into a 100-ml polypropylene container and sealed in place with epoxy resin. The container was closed with a tight-fitting lid carrying a centrally located pendant baffle. The test bacterium was suspended to 20–100 mg dry weight ml^{-1} in 50 ml of 0.1 M potassium phosphate buffer pH 6.9 placed inside the container and well agitated by a magnetic follower (rotating at 1,000 rpm). Anaerobiosis was maintained by sparging through the suspension a flow of oxygen-free N_2 delivered via a long wide-bore needle running through the lid to the base of the container. Incubation was at 30°C.

At intervals of approximately 1 h, 0.5 ml of the solvent mixture (with or without sulcatone at 9.5 mM) was injected into the stirred cell suspension until a total of 3 ml (approximately 6% v/v) had been added. Control runs were performed wherein no solvent additions were made. Output from the biomass probe was logged into a Personal Computer and analyzed essentially as previously described.[13]

Assay methods

Sulcatone (6-methylhept-5-en-2-one). This was performed by HPLC chromatography. A sample of supernatant obtained by centrifugation of the bacterial suspension (13,000 rpm for 4 min) was diluted with acetonitrile (typically about 15-fold) and 20 μl aliquots were applied to a 15 cm C-18 reverse phase column (Spherisorb ODS2) held at room temperature and eluted with a mobile phase of 30% (v/v) acetonitrile at a flow rate of 2 ml min^{-1}. The chromatography was performed using an LDC HPLC system (Laboratory Data Control, Riviera Beach, FL) with the UV absorbance at 210 nm of the eluate being continuously monitored with a LDC Spectromonitor III linked to a potentiometric chart recorder.

Sulcatol (6-methylhept-5-en-2-ol) enantiomeric excess (% e.e.). Usually three volumes (0.3 ml) of hexane were used to extract sulcatol from 1 ml samples of supernatant obtained, as above, from the bacterial suspensions; however, when sulcatone had been supplied at only very low concentration (e.g., 20 μM), the sulcatol was extracted with proportional amounts of hexane from 4-ml samples of supernatant. The hexane extracts were then combined and concentrated (to 0.5 ml) under a stream of N_2 gas. The sulcatol present in this concentrate was derivatized by the addition of 40 μl of N-trifluoroacetyl-(*S*)-(−)prolyl chloride (Aldrich Dorset, UK) and 50 μl of pyridine followed by incubation for 1 h at 72°C. After cooling, 0.1 ml of 1 M HCl was added, and the relative amounts of (*R*) and (*S*) sulcatol were determined by measurement by capillary gas chromatography of the respective concentrations of the diastereoisomers of the derivatives so produced. For this purpose, a Carlo Erba 9000 Series gas chromatograph (Carlo Erba Instruments, Rodano, Italy) was used after linking to a Hewlett Packard 3390A reporting integrator (Hewlett Packard, Avondale, PA). Samples (1 μl) were loaded onto a DB-1 column (15 m × 0.32 mm internal diameter) held at 170°C. The flame ionization detector and injection port were maintained at 250°C and the flow of carrier gas (He) was sustained at 2 ml min^{-1}. The enantiomeric excess (e.e.) was calculated, and expressed as a percentage using the equation,

$$\% \text{ e.e.} = 100 \times (R - S)/(R + S).$$

Results

Bioreductive enantioselectivity displayed by bacteria harvested at different stages of batch culture growth

It had previously been reported by Belan *et al.*[14] that whole cells of *C. tyrobutyricum* when harvested from the stationary phase of anaerobic batch culture would reduce sulcatone predominantly to (*R*)-sulcatol if glucose had been employed as the source of carbon and energy during their growth, but preferentially yielded the (*S*) enantiomer of sulcatol if growth had been achieved on crotonate in place of glucose. In the present study, these findings were reproduced when sulcatone (15 μl per 5 ml) was added to washed cell

Table 1 The enantiomeric purity of sulcatol produced from sulcatone by bacteria harvested at different times during batch culture on glucose or crotonate as carbon and energy source

	Enantiomeric excess (%)					
Bacterium	*C. tyrobutyricum* AuxP		*C. tyrobutyricum* PON5		*C. pasteurianum*	*L. brevis*
Carbon source	Glucose	Crotonate	Glucose	Crotonate	Glucose	Glucose
Time (h)						
6						84*R*
8						90*R*
12	11*S*	35*R*			86*R*	
14		40*R*	32*R*	39*R*		91*R*
15						92*R*
16		0			70*R*	
17	20*R*		18*R*	19*R*		90*R*
18					65*R*	
19	32*R*	50*S*				90*R*
20			20*S*	0	14*R*	
21	41*R*	70*S*				
23			41*S*	10*S*		
24	77*R*					90*R*
25			44*S*	17*S*	18*R*	

suspensions of strain AuxP of *C. tyrobutyricum* as long as the organisms had been harvested from the late stationary phase of batch culture; however, organisms harvested at the early exponential phase of batch culture growth behaved quite differently. Glucose-grown cells of *C. tyrobutyricum* strain AuxP taken from the start of the exponential phase thus produced sulcatol slight enriched (10% e.e.) in the (*S*)-enantiomer. The product contained portionately more of the (*R*)-enantiomer the longer the time of harvesting of the organisms from their batch culture medium was delayed (*Table 1*). When the same strain was grown in crotonate medium, cells harvested in early and mid exponential phase reduced sulcatone to yield sulcatol enriched in the (*R*)-enantiomer (40% e.e.); yet, suspensions consisting of cells that had been harvested later from this same crotonate batch culture demonstrated an increasing tendency to preference the formation of the (*S*)-enantiomer with organisms from the stationary phase of the culture yielding a product of nearly 80% e.e. (*S*)-sulcatol (*Table 1*). Identical results were obtained when similar bioreductions were undertaken using sulcatone dissolved in the novel solvent mixture and maintained in the cell suspensions at a concentration of only 20 μM (the maximum concentration of sulcatone in aqueous solution).[15]

Suspensions of *C. tyrobutyricum* PON5, a mutant strain selected for its ability to reduce ketones (including sulcatone) at enhanced specific rates,[9] had previously been shown to display no greater enantioselectivity in these reductions than did the parent strain (AuxP) from which it was derived,[9] however, organisms harvested at intervals during the progress of batch culture growth of strain PON5 demonstrated a gradually increasing tendency to preference the formation of (*S*)-sulcatol (from about 30–40% e.e. (*R*)-enantiomer produced by cells taken from mid exponential phase to 20–40% e.e. (*S*)-sulcatol formed by organisms harvested from the stationary phase; *Table 1*). This behavior differed from that of strain AuxP especially in that it was independent of whether glucose or crotonate had been the carbon and energy source during growth.

The enantioselectivity of sulcatone reduction by cells of *C. pasteurianum* grown on glucose was also dependent on the phase of batch culture growth at which they had been harvested. Sulcatol enriched in the (*R*)-enantiomer (80% e.e.) was produced by cells taken from the early exponential phase but this enrichment decreased as batch growth progressed until organisms from late exponential and stationary phase produced only 20% e.e. of the (*R*)-isomer (*Table 1*).

In contrast to the species of *Clostridium*, cell suspensions of *L. brevis* invariably produced (*R*)-sulcatol at about 90% e.e. irrespective of the phase of batch culture growth from which they had been harvested (*Table 1*).

The enantioselectivity of reduction of sulcatone can depend on the concentration at which the ketone is presented to the bacteria

To examine the effects of sulcatone concentration on the enantiomeric specificity of its reduction by the various bacterial suspensions, it was necessary to employ the solubilizing organic solvent mixture devised by Salter.[8] The potential toxicity of this mixture to the bacterial suspensions was therefore determined as described in the MATERIALS AND METHODS section. At the concentrations employed (which exceeded those to be used thereafter), the solvent mixture over a period of 7 h displayed no measurable toxicity to cell suspensions of *C. tyrobutyricum* AuxP. Similarly, following addition of the organic solvent mixture, a cell suspension of *L. brevis* NCIMB 947 in 7 h suffered a reduction in viability of only 1–2%. In contrast, even in the absence of the organic solvent mixture, the viability of a suspension of *C. pasteurianum* ATCC 6013 fell by approximately 11% in 7 h. In the presence of the organic solvent

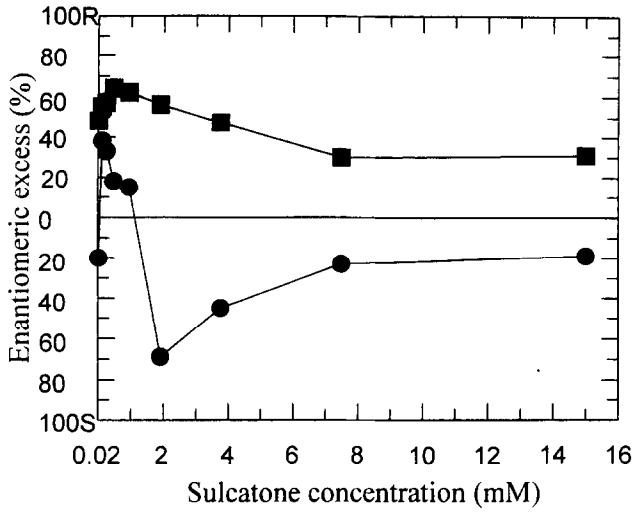

Figure 1 Effect of substrate (sulcatone) concentration on the enantiomeric purity of the sulcatol produced by cells of *C. tyrobutyricum* AuxP grown to late exponential phase of batch culture on glucose (■) or crotonate (●)

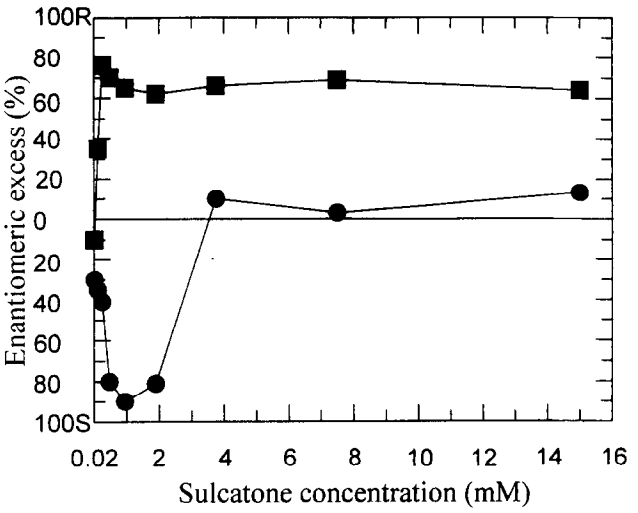

Figure 2 Effect of substrate (sulcatone) concentration on the enantiomeric purity of the sulcatol produced by cells of *C. tyrobutyricum* PON5 grown to late exponential phase of batch culture on glucose (●) or crotonate (■)

mixture, the decrease in viability was noticeable after some 3 h and was substantial (36%) at 7 h.

Whole cell suspensions were made using organisms harvested from the late expoential phase of batch culture growth and to these were added (under a H_2 atmosphere) sulcatone at various concentrations ranging from 20 μM to 15 mM. Suspensions of *C. tyrobutyricum* AuxP that had been grown on glucose yielded sulcatol enriched in the (*R*)-enantiomer irrespective of the concentration of sulcatone that was supplied to them (within the test range), although with increasing concentration of the ketone substrate above about 1 mM, there was a gradual decrease in the % e.e. of this product. On the other hand, the same organism grown to late exponential phase in crotonate medium although still producing (*R*)-sulcatol preferentially when sulcatone was provided at less than 1 mM at higher sulcatone concentrations predominantly formed the (*S*)-enantiomer (*Figure 1*). Mutant strain PON5 of *C. tyrobutyricum* grown to late exponential phase in batch culture on either glucose medium or crotonate medium gave a product somewhat enriched in (*S*)-sulcatol when sulcatone was supplied at 20 μM (*Table 1*). At increased concentrations of sulcatone, the crotonate-grown cells formed predominantly (*R*)-sulcatol (to a maximum of about 70% e.e.) while the glucose-grown cells produced (*S*)-sulcatol at 80–90% e.e. when supplied with sulcatone at 0.5 mM to 2 mM but above 4 mM sulcatone formed sulcatol that was only slightly enriched in the (*R*)-isomer (*Figure 2*). Suspensions of glucose-grown *C. pasteurianum* which when supplied with 20 μM sulcatone had formed sulcatol slightly enriched in its (*R*)-enantiomer increasingly formed higher proportions of the (*S*)-enantiomer as the substrate concentration was increased to about 2 mM; thereafter, over the range of sulcatone concentrations of 2–15 mM the product of its reduction was approximately 80% e.e. (*S*)-sulcatol (*Figure 3*).

In contrast to the behavior of the suspensions of clostridia, the washed cell suspension of *L. brevis* consistently produced 90% e.e. (*R*)-sulcatol over the complete test range of substrate sulcatone concentrations (*Figure 3*).

Discussion

Belan *et al.*,[14] when describing the manner in which the nature of the carbon and energy source employed for growth influenced the enantioselectivity of reduction of sulcatone that was displayed by *C. tyrobutyricum*, suggested that this might be attributable to the possession by the organism (in variable proportions) of at least two different reductases able to accept sulcatone as substrate. The present study demonstrated that the enantioselectivity of sulcatone reduc-

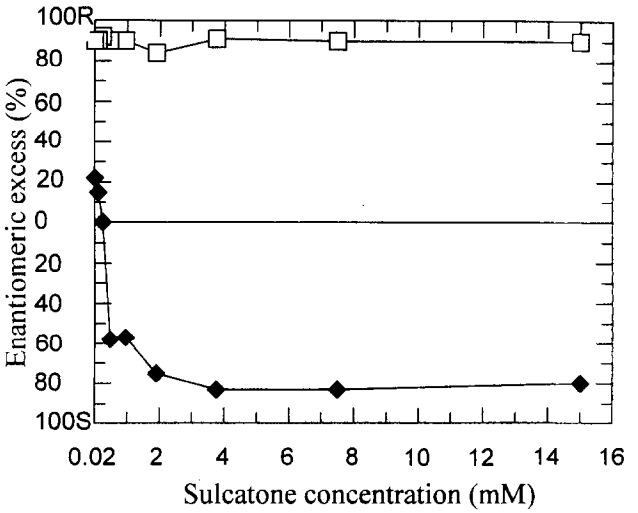

Figure 3 Effect of substrate (sulcatone) concentration on the enantiomeric purity of the sulcatol produced by cells of *C. pasteurianum* (◆) and *L. brevis* (□) grown to late exponential phase of batch culture on glucose media

tion by this organism also changed (a) as the organisms were harvested at different times during the course of batch culture growth and (b) when the concentration of sulcatone presented to the cells was altered. This could also be explained by the presence in the organism of two or more reductases that are synthesized in differing proportions during the course of batch culture growth and that differ in their relative activities (especially with respect to their values of k_{cat}/K_m with sulcatone). Indeed, Tidswell *et al.*[9] reported that *C. tyrobutyricum* PON5 possessed at least three constitutive ketone reductases that were easily separable by PAGE. Furthermore, partial purification of all three enzymes discerned that each exhibited quite distinct characteristics.[18] With this organism (*Figure 2*), it was possible by judicious choice of (i) the carbon and energy source employed during growth, (ii) the stage of batch culture growth at which the cells were harvested, and (iii) the concentration of sulcatone that was presented to the cell suspension to produce either (*R*)-sulcatol at 70% e.e. or (*S*)-sulcatol at 90% e.e.

A similar plurality of sulcatone reductases could explain the variability of the product when *C. pasteurianum* was the agent of bioreduction (*Table 1* and *Figure 3*). It might therefore be supposed that the invariability of the product when *L. brevis* catalyzed the reduction of sulcatone (*Table 1* and *Figure 3*) is due to this bacterium only possessing a single ketone reductase. This could also explain the high enantioselectivity displayed by this organism in its reduction of several other ketones;[16] however, partial purification of the sulcatone reductase activities from *L. brevis* indicated that this organism possesses at least five constitutive reductase enzymes.[17,18]

The findings made in the present study emphasize the importance of ensuring that, especially when cell suspensions of *Clostridium* sp. are to be employed as the biocatalyst, the manner of pregrowth of the organisms and the conditions under which the bioreduction is undertaken (including the concentration at which the substrate is supplied) are adjusted and controlled in such ways that will optimize production of the desired product enantiomer.

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