Journal of Molecular Catalysis B: Enzymatic 69 (2011) 15–20

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

New acetoin reductases from *Bacillus stearothermophilus: meso*- and 2*R*,3*R*-butanediol as fermentation products

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ARTICLE INFO

Article history:
Received 22 September 2010
Received in revised form 6 December 2010
Accepted 8 December 2010
Available online 15 December 2010

Keywords:
Fermentation
Bacillus stearothermophilus
Metabolic pathway
Diacetyl reductase
Acetoin reductase

ABSTRACT

The fermentation of sucrose with *Bacillus stearothermophilus* ATCC 2027 afforded 2*R*,3*R*- and *meso*-2,3-butanediol together with *R*-acetoin following the bacterial catabolic pathway. This metabolic route was confirmed by the conversion of pyruvate to *R*-acetoin with the cell free extract of *B. stearothermophilus*. On the other hand the reduction of 3*R*-acetoin to 2*R*,3*R*- and *meso*-butanediol with the same cell free extract allowed at least the presence of two NADH-dependent reductases. Together with the *S*-stereospecific diacetyl reductase (BSDR), purified in a previous work, a new *S*-stereospecific acetoin reductase (*S*-ACR) was partially purified and a fraction containing *R*-stereospecific acetoin reductase (*R*-ACR) was obtained. On the other hand in *B. stearothermophilus* fermentation of sucrose the "butanediol cycle" is negligible because the acetylacetoin synthase, responsible of the first step of this cycle, is an inducible enzyme that needs low concentration of sugar and high concentration of acetoin to be expressed.

1. Introduction

2,3-Butanediol is characterized by interesting properties and a wide range of applications [1]. Various bacteria are reported to produce this metabolite by fermentation [2] of simple sugars such as glucose and xylose (i.e., *Klebsiellla oxytoca* [3], *Enterobacter aerogenes* [4], *Bacillus polymyxa* [5], and *Bacillus licheniformis* [6]). The production of 2,3-butanediol is often coupled to the presence of other metabolites as 3-hydroxy-2-butanone (acetoin), ethanol, lactic acid and acetic acid [5]. On the other hand, 2,3-butanediol exists in three stereoisomeric forms [i.e., (−)-(2*R*,3*R*)- and (+)-(2*S*,3*S*)-butanediol, and the *meso*-form] and microorganisms generally give a mixture of *meso*-butanediol and one of the two enantiomers. Acetoin is the main precursor of 2,3-butanediol and is formed in bacteria from pyruvate (catabolic pathway) by action of two enzymes: (i) α-acetolactate synthase, that catalyzes the condensation of two molecules of pyruvate with a single decarboxylation to afford α-acetolactate; (ii) α-acetolactate decarboxylase that decarboxylates this last one to acetoin [7].

The different isomeric forms of butanediol can be produced by acetoin reduction with various acetoin reductases with different stereospecificity [8] or by a cyclic pathway called "butanediol cycle" which existence has been reported in different bacteria [9] (Scheme 1).

In our previous works *Bacillus stearothermophilus* ATCC 2027 was efficiently used in the kinetic resolution *via* oxidation of racemic alcohols [10,11]. A partially purified dehydrogenase was also obtained able to catalyze the enantioselective reduction of the corresponding prochiral ketones [12]. Successively this enzyme was fully purified, characterized as diacetyl(acetoin)reductase (BSDR) [13] and used in the reduction of various α-diketones to produce enantiomerically pure *S*,*S*-diols and *S*-α-hydroxyketones [14].

In the present work, the fermentation of sucrose with *B. stearothermophilus* to 3*R*-acetoin, 2*R*,3*R*- and *meso*-butanediol and the role of two new NADH-dependent acetoin reductases (i.e., *S*-stereospecific and *R*-stereospecific) in their formation were described.

2. Experimental

2.1. Chemicals and culture media

B. stearothermophilus ATCC 2027 was purchased from the American Type Culture Collection (ATCC). Bacto tryptone and yeast extract (Oxoid), glucose-6-phosphate dehydrogenase (G6PD) (Sigma), (+)-2*S*,3*S*-butanediol, (−)-2*R*,3*R*-butanediol, *meso*-butanediol and *R*/*S* acetoin are commercially available.

2.2. GLC analysis

GLC analyses were performed on a Carlo Erba 6000 equipped with a fused capillary column Megadex 5 (25 m × 0.25 mm) containing dimethyl-*n*-pentyl-β-cyclodextrin on OV 1701 (from Mega

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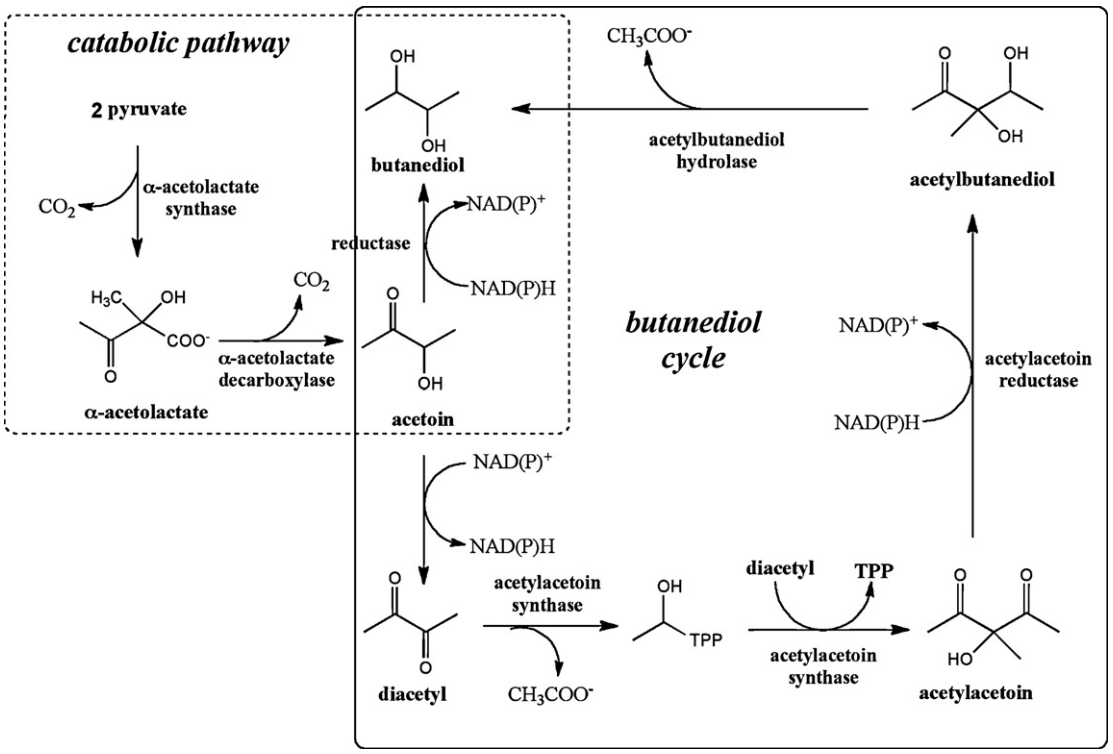
doi:10.1016/j.molcatb.2010.12.004



Scheme 1. Metabolic pathways to 2,3-butanediol.

snc) using acetophenone as internal standard and helium as carrier gas (80 kPa); temp. 90–200 °C (2 °C/min); retention times (min): (−)-3*R*-acetoin, 2.95; (+)-3*S*-acetoin, 3.10; acetylacetoin, 4.36; acetylbutanediol, 4.40; (+)-2*S*,3*S*-butanediol, 5.90; (−)-2*R*,3*R*-butanediol, 6.20; *meso*-butanediol, 6.60; acetophenone 11.30.

2.3. Fermentation of sucrose with B. stearothermophilus

B. stearothermophilus ATCC 2027, maintained at −196 °C as CryoVials glycerol stocks in TY medium, was cultured in a 500 ml Erlenmeyer flask with 100 ml of a medium containing sucrose (30 g/l), peptone (20 g/l), yeast extract (10 g/l), $Na_2HPO_4{\cdot}6H_2O$ (6.8 g/l), K_2SO_4 (2.6 g/l), and $MgSO_4$ (0.3 g/l), at 38–39 °C. After 24 h incubation in a shaking incubator (110 rpm), 5 ml of this culture were harvested under sterile condition and used as inoculum for the fermentation.

The fermentation was set up in 500 ml Erlenmeyer flask with 100 ml of the same medium. Aliquots were withdrawn periodically and monitored by GLC. Each sample (2 ml) was centrifuged (6000 rpm, 10 min) and NaCl (0.2 g) was added to the supernatant that was successively extracted with ethyl acetate (0.8 ml) containing 0.1% (v/v) of acetophenone as internal standard. The time course study of sucrose (30 g/l) was described in Fig. 1.

2.4. Preparation of the cell free extract

B. stearothermophilus was cultivated in the same medium (500 ml) described for the fermentation inoculum at 39 °C. After 48 h the cells (4 g) were harvested by centrifugation (7000 rpm, 10 min), washed with 0.15 mM NaCl (50 ml) and suspended in 50 mM triethanolamine buffer (TEA buffer) at pH 7.5 (20 ml) containing β-mercaptoethanol (1 mM) and EDTA (0.1 mM). Phenylmethylsulfonyl fluoride (1 mM) was added and the suspension was treated at high pressure (1380 bar) with a French press and then centrifuged (15,000 rpm, 20 min, 5 °C). The supernatant cell free extract (18 ml) was used to catalyze the synthesis of *R*-acetoin, the reduction of racemic acetoin, and as starting material for the dehydrogenases separation.

2.5. Biosynthesis of 3R-acetoin

The cell free extract (4 ml) was added to a solution of sodium pyruvate (0.1 g, 0.9 mmol) in 50 mM phosphate buffer at pH 6.0 (100 ml). After 2 h at 30 °C the reaction mixture was saturated with NaCl and extracted with ethyl acetate (3× 20 ml). The organic layer was dried over anhydrous Na_2SO_4 and analyzed by GLC using the above reported conditions. A single peak at 2.95 min was detected corresponding to (−)-3*R*-acetoin. The stereochemistry of acetoin was confirmed by its reduction with $NaBH_4$. Ethyl acetate was removed under reduced pressure, and the residue was dissolved in diethyl ether/methanol 5:1 (20 ml). The mixture was cooled (ice bath) and $NaBH_4$ (35 mg, 0.9 mmol) was added. After 30 min the mixture was diluted with water (10 ml), the organic layer was separated, dried over anhydrous Na_2SO_4 and analyzed by GLC. 2*R*,3*R*-Butanediol and *meso*-butanediol were obtained (35% and 60% yield, respectively) and no 2*S*,3*S*-butanediol was detected (Scheme 2, A).

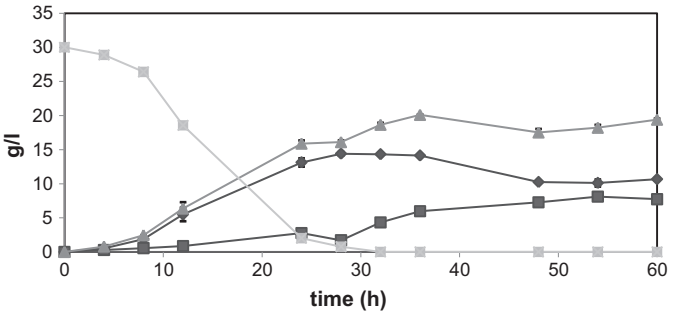
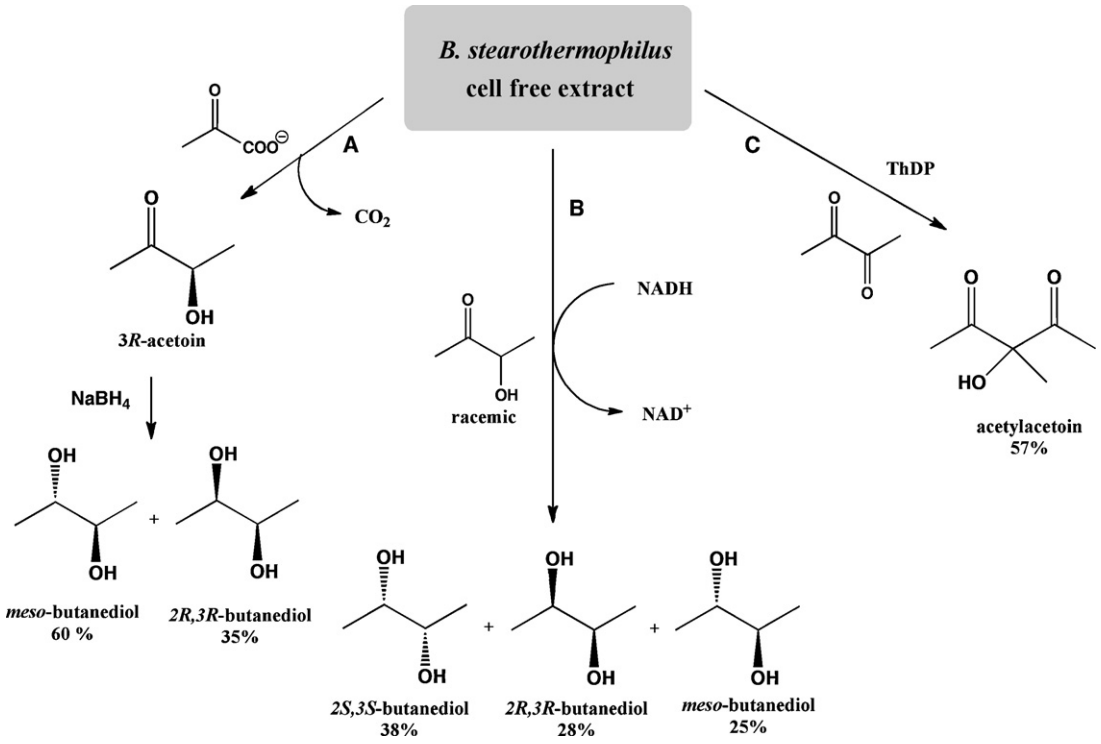


Fig. 1. Time course study of sucrose (30 g/l) fermentation by *B. stearothermophilus*: (■) sucrose; (■) acetoin; (♦) butanediol;(▲) butanediol + acetoin.



Scheme 2. Biotranformations with *B. stearothermophilus* cell free extract: (A, B), *B. stearothermophilus* grown on sucrose; (C) *B. stearothermophilus* grown on acetoin.

2.6. Bioreduction of racemic acetoin

The cell free extract (0.1 ml), obtained as above described, was added to a solution of racemic acetoin (3 mg, 34 μmol) and NADH (50 mg, 70 μmol) in 50 mM phosphate buffer at pH 6.5 (3 ml). After 1 h the reaction mixture was saturated with NaCl and extracted with ethyl acetate (3× 1 ml). The organic layer was dried over anhydrous Na_2SO_4 and analyzed by GLC using acetophenone as internal standard. (+)-2*S*,3*S*-Butanediol, (−)-2*R*,3*R*-butanediol and *meso*-butanediol have been obtained in 38, 28 and 25% yield, respectively (Scheme 2, B).

2.7. Synthesis of acetylacetoin with acetoin-induced cell extract

B. stearothermophilus was cultured in a medium (200 ml) containing meat extract (10 g/l), polypeptone (10 g/l), NaCl (5 g/l) and acetoin (5 g/l). After 48 h growth at 39 °C the cells (2 g, wet weight) were harvested by centrifugation (6000 rpm, 10 min), washed with 150 mM NaCl solution (50 ml) and suspended in 50 mM phosphate buffer at pH 6.5 (50 ml). The suspension was treated at high pressure (1380 bar) with a French press and then centrifuged (15,000 rpm, 20 min, 5 °C). To the supernatant (46 ml), used without further purification to catalyze the coupling reactions, diacetyl (0.26 g, 3 mmol), thiamine diphosphate (ThDP) (15 mg, 35 μmol), and magnesium sulphate (10 mg, 83 μmol) in 50 mM phosphate buffer at pH 6.5 (50 ml) were added and acetylacetoin was obtained as described [15] (Scheme 2, C).

The addition of diacetyl to the cell extract obtained on sucrose did not afford acetylacetoin.

2.8. Separation of B. stearothermophilus acetoin reductases

The enzyme separation was followed measuring the absorbance variation (340 nm) during the acetoin reduction. A few μl of the eluted fractions were added to a solution of acetoin (5 mM) and NADH (0.2 mM) in 50 mM phosphate buffer at pH 6.5 (1 ml). One unit (U) is defined as the enzyme amount able to catalyze the reduction of 1 μmol of acetoin in one minute. The cell free extract (10 ml) was loaded on a DEAE–sepharose column (1.5 cm × 3 cm) equilibrated with a 50 mM TEA buffer at pH 7.5 containing 1 mM β-mercaptoethanol and 0.1 mM EDTA. The column was washed until no proteins were present in the effluent. This first fraction (11 ml, 50 U), contains *B. stearothermophilus* diacetyl(acetoin)reductase (BSDR) that can be purified as described [13]. After washing DEAE–sepharose column with 0.1 M NaCl in TEA buffer, the *S*-stereospecific acetoin reductase (*S*-ACR) was eluted with 0.2 M NaCl (8 ml, 25 U). Finally washing the column with 0.4 M NaCl the *R*-stereospecific acetoin reductase (*R*-ACR) was eluted (8 ml, 7 U) (Fig. 2).

The fraction containing *S*-ACR was partially purified adding NaCl in order to obtain a 1.5 M final concentration and loading the solution on a phenyl–sepharose column (0.5 cm × 4 cm) equilibrated with 1.5 M NaCl in TEA buffer at pH 7.5 containing 1 mM β-mercaptoethanol and 0.1 mM EDTA. The column was eluted with a linear NaCl gradient (from 1.5 to 0.5 M) and the active fractions were collected (5 ml, 20 U).

2.9. Enzymatic reduction of racemic acetoin. General procedure

Each of the three different enzymatic fractions eluted from DEAE–sepharose (0.1 ml) was added to a solution of racemic acetoin (3 mg, 34 μmol) and NADH (50 mg, 70 μmol) in 50 mM phosphate buffer at pH 6.5 (3 ml). After 12 h the reaction mixtures were analyzed by GLC as previously described for the fermentation. The results are reported in Table 1.

2.10. Enzymatic oxidation of meso-, 2R,3R- and 2S,3S-butanediol. General procedure

The appropriate enzymatic fraction (0.2 ml) was added to a 50 mM TEA buffer solution at pH 8.2 (3 ml) containing the proper butanediol isomer (3 mg, 34 μmol) and NAD^+ (50 mg, 70 μmol).

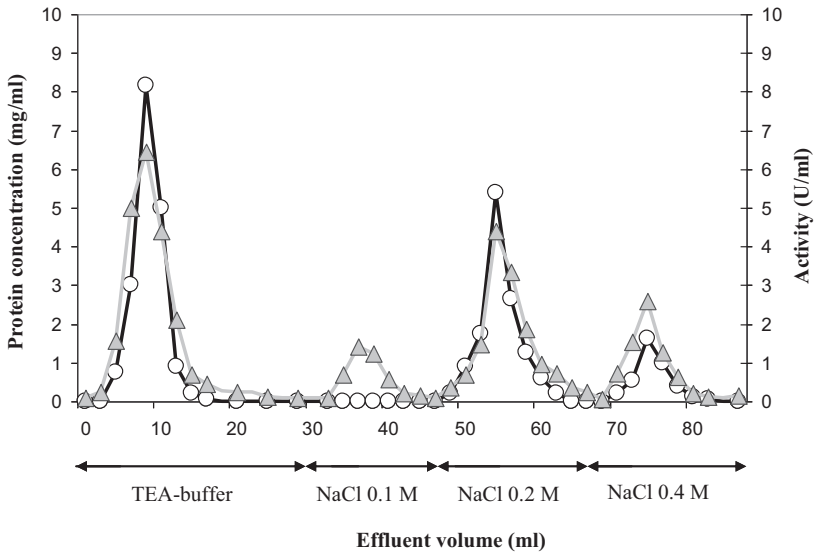


Fig. 2. Chromatographic separation of *Bacillus stearothermophilus* reductases: (○) activity; (▲) protein concentration.

After 12 h the reaction mixture was analyzed by GLC as previously described for the fermentation. The results are summarized in Table 1.

2.11. Enzymatic reduction of acetylacetoin. General procedure

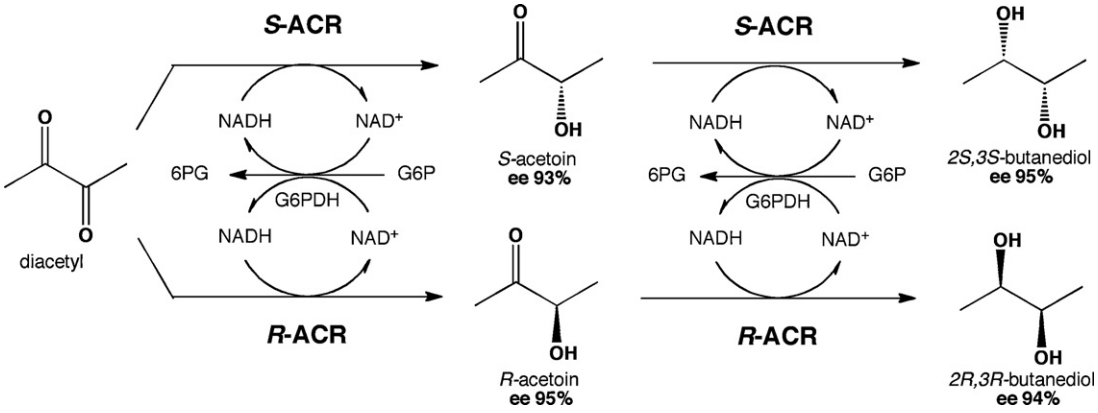
Each of the three different enzymatic fractions eluted from DEAE–sepharose column (1 ml) was added to a solution of acetylacetoin (8 mg, 60 μmol) and NADH (100 mg, 140 μmol) in 50 mM phosphate buffer at pH 6.5 (6 ml). After 12 h the reaction mixture was extracted with ethyl-acetate (3× 5 ml) and the organic layer, dried over anhydrous sodium sulphate, was evaporated. The crude products were analyzed by GLC and ^{1}H NMR. Acetylacetoin was converted to acetylbutanediol (95%, ee > 95%) by BSDR while the reactions with *S*-ACR and *R*-ACR did not give any new product (Scheme 4). Acetylbutanediol showed the following: ^{1}H NMR, $CDCl_3$, ^{TM}: 4.05 (q, 1H, J = 7.5 Hz, CHOH), 2.3 (s, 3H, CH_3CO), 1.3 (d, 3H, J = 7.5 Hz, CH_3), 1.3 (s, 3H, CH_3).

2.12. S-ACR-catalyzed synthesis of 3S-acetoin and 2S,3S-butanediol

Partially purified *S*-ACR (1.3 ml, 5.2 U) was added to a solution of diacetyl (0.1 g, 1.16 mmol), NAD^+ (5 mg, 7 μmol), glucose-6-phosphate (G6P) (0.37 g, 1.3 mmol) and glucose-6-phosphate dehydrogenase (G6PDH) (25 μg, 15 U) in 50 mM phosphate buffer at pH 6.5 (25 ml). The solution was gently shaken at 25 °C for 8 h, than saturated with NaCl and extracted with ethyl acetate

Table 1
Enzymatic reactions of acetoin and butanediol with *B. stearothermophilus* reductases.

Substrate	Enzyme/*cofactor*	Products Yield %				
		 *2R,3R*	 *2S,3S*	 *meso*	 *3R*	 *3S*
 racemic	**BSDR**/*NADH*	--	38	42		
	***S*-ACR**/*NADH*	--	30	41		
	***R*-ACR**/*NADH*	37	--	25		
 *meso*	**BSDR**/NAD^+				68	--
	***S*-ACR**/NAD^+				61	--
	***R*-ACR**/NAD^+				--	56
 *2R,3R*	**BSDR**/NAD^+				--	--
	***S*-ACR**/NAD^+				--	--
	***R*-ACR**/NAD^+				52	--
 *2S,3S*	**BSDR**/NAD^+				--	61
	***S*-ACR**/NAD^+				--	--
	***R*-ACR**/NAD^+				--	--



Scheme 3. *S*-ACR and *R*-ACR reduction of diacetyl: G6PDH, glucose-6-phosphate dehydrogenase; G6P, glucose-6-phosphate, 6PG, 6-phosphogluconic acid.

(3× 20 ml). The organic layer was dried over anhydrous sodium sulphate and evaporated. Chromatography of the residue on silica gel (diethyl ether/cyclohexane 1:1 as eluent) gave 3*S*-acetoin (72 mg, 71%, ee 93%).

The same reaction, repeated doubling glucose-6-phosphate (0.74 g, 2.6 mmol) and following the same work-up, after 15 h afforded 2*S*,3*S*-butanediol (63 mg, 60%, ee 95%) (Scheme 3).

2.13. *R*-ACR-catalyzed synthesis of 3*R*-acetoin and 2*R*,3*R*-butanediol

Partially purified *R*-ACR (5.7 ml, 5 U) was added to a solution of diacetyl (0.1 g, 1.16 mmol), NAD^+ (5 mg, 7 μmol), glucose-6-phosphate (G6P) (0.37 g, 1.3 mmol) and glucose-6-phosphate dehydrogenase (G6PDH) (25 μg, 15 U) in 50 mM phosphate buffer at pH 6.5 (20 ml). The solution was gently shaken at 25 °C for 10 h, then saturated with NaCl and extracted with ethyl acetate (3× 20 ml). The organic layer was dried over anhydrous sodium sulphate and evaporated. Chromatography of the residue on silica gel (diethyl ether/cyclohexane 1:1 as eluent) gave 3*R*-acetoin (68 mg, 66%, ee 95%).

The same reaction, repeated doubling glucose-6-phosphate (0.74 g, 2.6 mmol) and following the same work-up, after 20 h afforded 2*R*,3*R*-butanediol (57 mg, 55%, ee 94%) (Scheme 3).

3. Results and discussion

B. stearothermophilus ATCC 2027 fermentation of sucrose (30 g/l) afforded 2*R*,3*R*-butanediol (ee 80–95%) and *meso*-butanediol ($Y_{p/s}$ 0.56) together with variable amounts of *R*-acetoin (ee 80–95%). Other sugars were also tested with no appreciable results [16].

In Fig. 1 the time course of 30 g/l sucrose fermentation was reported. At 32 h practically no sucrose was detected and the higher concentration of butanediol was produced. After this time the concentration of butanediol decreased and a higher production of acetoin was obtained even if the total amount of butanediol and acetoin was practically constant.

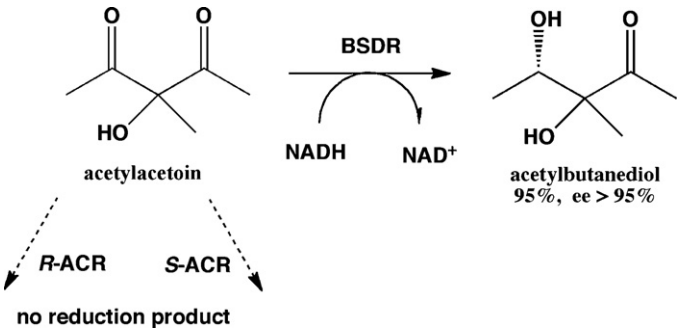
The metabolic pathway for the main butanediol forming bacteria deals with the conversion of α-acetolactic acid derived from pyruvic acid to acetoin in the catabolic butanediol route (Scheme 1). The presence of this pathway in *B. stearothermophilus* ATCC 2027 was confirmed by the formation of acetoin when freshly prepared cell free extract was added to a solution of sodium pyruvate (Scheme 2, A).

Only one enantiomer of acetoin was detected (ee > 95%) and its configuration was deduced on the basis of the butanediol isomers formed by its reduction with $NaBH_4$. This reduction afforded a mixture of 2*R*,3*R*-butanediol (35%) and the *meso* form (60%) proving that *B. stearothermophilus* produced the *R*-acetoin from pyruvate according to the other butanediol forming bacteria [17].

Moreover since the same mixture of *meso*- and 2*R*,3*R*-butanediol was produced by *B. stearothermophilus* fermentation of sucrose and since these stereoisomers arise from the same precursor *R*-acetoin we have at least hypothesized the existence of two acetoin reductases according to the catabolic pathway: an *S*-stereospecific acetoin reductase to produce the *meso*-butanediol and an *R*-stereospecific acetoin reductase to have 2*R*,3*R*-butanediol. This hypothesis was further supported by the reduction of the racemic acetoin with *B. stearothermophilus* cell free extract in the presence of NADH. The reduction afforded 2*R*,3*R*-butanediol in 28% yield together with the 2*S*,3*S*-butanediol and *meso* form (38% and 25% yield, respectively) (Scheme 2, B). On the other hand no reduction product was obtained in the presence of NADPH.

On the other hand, regarding the possible production of butanediol following the "butanediol cycle", this hypothesis is negligible because the acetylacetoin synthase is an inducible enzyme that needs low concentration of sugar and high concentration of acetoin to be expressed [9]. In fact the cell free extract of *B. stearothermophilus*, grown on acetoin, produced acetylacetoin starting from diacetyl (Scheme 2, C) as reported in a previous work [15] while the cells free extract obtained on sucrose did not catalyze the conversion of diacetyl to acetylacetoin.

In our previous work we described the purification and characterization of a diacetyl(acetoin)reductase (BSDR) from *B. stearothermophilus* [13]. This NADH-dependent enzyme showed an *S*-enantioselectivity in the reversible reduction of acetoin so it could be responsible of the *meso*-butenediol formation from *R*-acetoin. The reported BSDR purification procedure was characterized by the loss of about 50% of the original activity (determined measuring the rate of racemic acetoin reduction) during the first chromatographic step on diethylaminoethyl–sepharose. BSDR was not bounded by the resin and was eluted by the equilibration buffer. This step was repeated and subsequently the column was washed with increasing concentration of NaCl in TEA buffer at pH 7.5. Other two active enzymatic fractions were eluted with 0.2 and 0.4 M NaCl, respectively (Fig. 2). The fraction eluted with 0.2 M NaCl was further purified by hydrophobic interaction chromatography on phenyl–sepharose. This step allowed a purification of 6.5 times and a recover of 81% (it is not possible to determine an overall value of purification because no selective assay method for BSDR and *S*-ACR are available). The enzymatic stereospecificity was investigated by adding each fraction to a solution of racemic acetoin and NADH in phosphate buffer at pH 6.5 and analyzing after 12 h the reaction mixture by GLC. The results are reported in Table 1.



Scheme 4.

The enzymatic fractions eluted with 0.2 M NaCl afforded a mixture of 2*S*,3*S*-butandiol and the *meso* form (30 and 41%, respectively) and were named *S*-stereospecific acetoin reductase (*S*-ACR).

Moreover, the enzymatic fractions eluted from DEAE–sepharose with 0.4 M NaCl gave 2*R*,3*R*-butanediol (37%) together with the *meso* form (25%) and were named *R*-stereospecific acetoin reductase (*R*-ACR). On the other hand BSDR gave a mixture of 2*S*,3*S*- and *meso*-butanediol (38 and 42%, respectively).

The specificity of the above dehydrogenases was further investigated in the oxidation of the butanediol stereoisomers with NAD^+ as cofactor (Table 1). The BSDR and *S*-ACR catalyzed efficiently the oxidation of *meso*-butanediol affording 3*R*-acetoin (68 and 61% yield, respectively). Also the *R*-ACR oxidized the *meso* stereoisomer giving 3*S*-acetoin in 56% yield. On the other hand, only BSDR was able to catalyze the oxidation of 2*S*,3*S*-butanediol affording 3*S*-acetoin in 61% yield. Moreover, only *R*-ACR oxidized 2*R*,3*R*-butanediol to 3*R*-acetoin (52%).

These results demonstrated the presence of a pool of dehydrogenases able to explain the formation of 2*R*,3*R*- and meso-butanediol following the catabolic pathway.

The synthetic potential of these new stereospecific acetyl reductases was verified in the reduction of diacetyl. *S*-ACR afforded stereospecifically 3*S*-acetoin (71%, ee 93%) or 2*S*,3*S*-butanediol (60%, ee 95%) using different amount of glucose-6-phosphate dehydrogenase (G6PDH)/glucose-6-phosphate (G6P) to recycle the NADH (Scheme 3). Similar behaviour has shown *R*-ACR producing 3*R*-acetoin (66%, ee 95%) or 2*R*,3*R*-butanediol (55%, ee 94%) in the same conditions.

On the other hand the existence of the acetylacetoin synthase [15], responsible of the first step of the "butanediol cycle", and of two S-stereospecific dehydrogenases (i.e., BSDR and S-ACR) prompted us to verify the dehydrogenases activity towards acetylacetoin.

Only BSDR was able to reduce acetylacetoin to acetylbutanediol, characterized by NMR, in almost quantitative yield (ee > 95%) (Scheme 4). On the basis of this result it is possible to assume that the physiologic role of BSDR in "butanediol cycle" is to catalyze the conversion of acetylacetoin to acetylbutanediol.

4. Conclusions

The fermentation of 30 g/l sucrose with *B. stearothermophilus* to 2,3-butanediol and acetoin (coefficient yield 0.56) was reported. The metabolic pathway to the fermentation products was also studied and was possible to assert that *B. stearothermophilus* produced 3*R*-acetoin following the "catabolic pathway." This metabolite was the precursor of *meso*- and 2*R*,3*R*-butanediol and two new acetoin reductases (i.e., *S*-ACR and *R*-ACR) were separated together with the previously reported BSDR. BSDR and *S*-ACR catalyzed the reduction of *R*-acetoin to *meso*-butanediol, while its reduction with *R*-ACR produced 2*R*,3*R*-butanediol. In these fermentation conditions the "butanediol cycle" was negligible because acetylacetoin synthase was not expressed.

These pool of acetoin reductases are potentially useful for the biocatalytic approach to the synthesis of *S*- and *R*-stereospecific optically active α-hydroxyketones and *vic*-diols.

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