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Glycerol Effect on Spiramycin Production and Valine Catabolism in *Streptomyces ambofaciens*

Anissa Lounès, Ahmed Lebrihi, Chouki Benslimane, Gérard Lefebvre, Pierre Germain

Laboratoire de Microbiologie Industrielle et Alimentaire, ENSAIA, Institut National Polytechnique de Lorraine, 2 Avenue de la Forêt de Haye, 54500 Vandoeuvre, France

Abstract. Spiramycin production by *Streptomyces ambofaciens* in a chemically defined medium, with valine as nitrogen source, was controlled by the nature and the concentration of the carbon source. The production of this antibiotic was better in dextrins than in glycerol-containing medium. The negative effect of glycerol could be attributed in part to an excess of energy and a high specific growth rate. The intracellular ATP content, at the start of spiramycin production, was twofold higher in glycerol than in dextrin-containing medium. Increasing the initial concentrations of glycerol led to an increase in the specific growth rate and a drop in spiramycin production. Comparison between glycerol and a protein synthesis inhibitor effects and the use of resting cell systems (RCS) proved that glycerol exerted both inhibitory and repressive actions on spiramycin production independently from the growth. At the enzymatic level, glycerol interfered with valine catabolism by repressing partially valine dehydrogenase (VDH) and α-ketoisovalerate dehydrogenase (KIVDH), generator of spiramycin aglycone precursors.

Streptomyces ambofaciens produces a macrolide antibiotic, spiramycin. Among the constituents of this molecule is a 16-membered branched lactonic ring that requires eight precursors for its biosynthesis: five acetate, one propionate, one butyrate, and another unidentified precursor, made up of two carbons as precursors for its biosynthesis [16]. These latter compounds are final products of some pathways of primary metabolism. They are synthesized from glycolysis, the tricarboxylic acid cycle, and the catabolism of fatty acids and/or amino acids; thus they occupy a key position between primary and secondary metabolism. The polymerization of activated acyl units to form the macrolide lactonic ring is catalyzed by a polyketide synthetase enzyme system, similar to the events leading to the synthesis of long-chain fatty acids [15].

Valine is deaminated and decarboxylated to give isobutyrate, which is probably isomerized to *n*-butyrate, precursor of platenolide biosynthesis [17, 18]. Valine dehydrogenase (VDH), the first enzyme set in work in the valine catabolism pathway, led to the production of ammonium and α-ketoisovalerate. This oxidative deamination conferred to valine its role of nitrogen source. Later steps allowed the degradation of α-ketoisovalerate until succinyl-CoA supplying TCA cycle. Therefore α-ketoisovalerate could play the role of carbon and energy source. This catabolism provided to the cell the principal intermediate precursors of the macrolide aglycone (butyryl-CoA, propionyl-CoA, . . .).

Nitrogen regulation was studied in part in *S. ambofaciens*. Thus, it was demonstrated that biosynthesis of the 16-membered macrolide spiramycin was inhibited by a high concentration of ammonium and stimulated by the catabolism of certain amino acids [10, 20]. Ammonium ions suppressed spiramycin aglycone biosynthesis by interfering with valine catabolism. Synthesis of valine dehydrogenase was repressed by an excess of ammonium ions [10].

Whereas carbon control of spiramycin biosynthesis still remains unknown, biosynthesis of several antibiotics was subject to regulation by a rapidly assimilated carbon source. Although this control was described since 1950s, the mechanisms involved and the targets of a such control are known only in a few cases [6, 7, 13]. For example, expandase activity in *S.*

Correspondence to: A. Lebrihi

clavuligerus was inhibited by some phosphorylated intermediates of carbon catabolism pathway [9]. It was shown that the formation of the deacetoxycephalosporin C synthase ("expandase") in *Cephalosporium acremonium* was markedly repressed by growth on glucose, glycerol, or maltose [13].

We demonstrated in a previous study that valine served as nitrogen, carbon, and energy sources [11]. In the present study, the effect of dextrins and glycerol, slowly and rapidly assimilated carbon sources respectively, on spiramycin production and valine catabolism in *S. ambofaciens* was investigated. At the enzymatic level, the regulation by glycerol of two enzymes of valine catabolism (suppliers of spiramycin aglycone precursors) was examined: valine dehydrogenase (VDH), which provided the nitrogen necessary to the cell, and α-ketoisovalerate dehydrogenase (KIVDH), which used valine as carbon and energy source and as a generator of spiramycin aglycone precursors.

Materials and Methods

Microorganisms. These studies were carried out with *Streptomyces ambofaciens* RP 181110 (Rhône-Poulenc, Vitry-sur-Seine, France), a producer of spiramycin, and *Bacillus subtilis* ATCC 6633 as a control microorganism.

Medium and culture conditions. Spores of *S. ambofaciens* were maintained in 20% glycerol at −20°C; 0.1 ml of this suspension was used to inoculate 50 ml of the seed medium and incubated for 48 h. The resulting seed culture was inoculated into the desired fermentation medium (4% vol/vol). All liquid cultures were conducted in 300-ml Erlenmeyer flasks containing 50 ml of medium, at 28°C on a rotatory shaker at 250 rpm. The basal medium, developed in our laboratory, contained per liter 1 g $MgSO_4$, 15 mg $ZnSO_4$, 2 g KH_2PO_4, 5 g $CaCO_3$, 20 g NaCl, 0.3 mg $CoCl_2$. The concentration and nature of nitrogen and carbon sources added to the basal medium are described in the text.

All the cultures or experiments were done at least three times. In each culture, all the assays were performed at least in triplicate and the mean values presented; they gave results within 5% of each other. Variation in cell dry weight (and glycerol, dextrins, valine, ammonium ions, spiramycin, VDH, and KIVDH activities) among triplicate experiments was within 10%.

Resting cell experiments. Cells grown for 48, 72, or 96 h in defined medium were harvested by centrifugation (8000 *g*, 10 min, 4°C), washed twice with 10 mM MOPS buffer (pH 6.9), and resuspended in the same buffer. A portion of the washed mycelia was taken for cell dry weight (CDW) measurement, and the remainder was resuspended in the basal medium described above with dextrins without nitrogen source (MWN), to achieve a mycelial density of approximately 3 g · L^{-1} of suspension. To 15 ml of the mycelial suspension in a 100-ml flask, a 5 ml supplement was added. The supplement consisted of buffer or an aqueous carbon source solution (final concentration of glycerol was 60 g · L^{-1}) or an aqueous streptomycin solution (final concentration of inhibition was 100 mg · L^{-1}). The incubation conditions were the same as those for fermentation culture. Samples (1 ml) were withdrawn periodically for antibiotic measurement. The kinetics of spiramycin production were followed all through the fermentation, and the specific spiramycin productions were calculated.

Isolation of streptomycin-resistant mutant of *B. subtilis* ATCC 6633. A suspension of *B. subtilis* was spread on agar plates containing 30 mg · L^{-1} of streptomycin and incubated at 37°C. Colonies were picked after 2 days of incubation and streaked out on nutrient broth supplemented with 30 mg · L^{-1} of streptomycin for enrichment. Series of progressive passages on nutrient plates from 30 to 100 mg · L^{-1} streptomycin, inserted with inoculation on liquid broth at every stage for enrichment, were carried out to obtain streptomycin-resistant mutant of *B. subtilis*. Colonies resistant to 100 mg · L^{-1} of streptomycin were picked, and their sensitivity to spiramycin was tested. The one which gave the sharpest inhibition zone and superimposable standard curves in the presence or absence of 100 mg · L^{-1} streptomycin was chosen and called *B. subtilis* (Brs).

Growth measurement. Determination of cell dry weight (CDW) was performed as described by Lebrihi and associates [8]. Under the conditions used, 1 g CDW · L^{-1} corresponded to an optical density (OD) of 2 at 660 nm.

Dextrins and glycerol determination. Dextrins and glycerol in the medium were determined as described by Hanson and Phillips [4] and Bok and Demain [2] respectively.

Ammonium determination. The concentration of ammonium, in the culture broth was determined by using an ammonia electrode (Orion Research, Boston, MA, USA) with ammonium chloride as standard.

Intracellular ATP content. ATP was measured by the luciferase assay as described by Jakubczak and Leclerc [5].

Valine determination. Thin-layer chromatography (TLC), silica gel 60 plates (20 × 20 cm) were used for quantitative analysis of valine in the medium. We used *n*-butanol–acetic acid–water (3:1:1, vol/vol) as the migration solvent system and ninhydrin for the revelation. The quantification of valine was performed by densitometry (Shimadzu CS 9000 from Roucaire, Vélizy-Villacoublay, France). A linear relationship between valine concentration and peak area was obtained for amounts in the range of 0.1 to 1 g · L^{-1} of valine.

Short-chain fatty acid measurements. Short-chain fatty acids in the bacterial culture were quantified by gas chromatography (IGC 121 model EL, INTERSMAT) with a propak Q80, 100-mesh (2 m × 2 mm) gas chromatograph and a flame ionization detector. The column temperature was 180°C. Known amounts of standard lower fatty acids were used for calibration procedures (external standard). Eight microliters of external standard or supernatant of sample was mixed with 2 ml of internal standard (prepared with 4 ml of methanol plus 6 ml of 6 M HCl in a final volume of 100 ml with distilled water). Two microliters of the resulting mixture was injected for analysis.

Spiramycin determination. The spiramycin content was determined by the conventional disc diffusion method with *B. subtilis* as the sensistive strain. Triplicate assays were done; the variation was less than 10%. Under our culture conditions, the purified spiramycin I was the majority fraction. The ratio of the different fractions (I, II and III) of spiramycin was assayed by HPLC (see below).

Preparation of crude cell-free extracts. The mycelia obtained by filtration were washed twice with distilled water and then resuspended in 15 ml of 0.05 M Tris-HCl buffer (pH 8.0) containing 30%

glycerol. The mycelia were disrupted in an ice-water bath with a sonicator (Ultrasonics Inc.; model W225 R) for 5 min at 120 W. Cell fragments were removed by centrifugation at 12,000 *g* for 30 min. The supernatant fluid was used as enzymatic extract. The protein concentration of cell-free extracts was estimated by the method of Lowry and colleagues [12], with bovine serum albumin as the standard.

Enzyme activities. These enzyme activities were determined from at least two cultures in each growth condition. For each culture, three time points were taken to establish the rate of enzymic reaction. The mean values of all these assays were calculated. The variation was no more than 15%.

Valine dehydrogenase (VDH). VDH activity was assayed according to the method described by Schütte and coworkers [19], originally described for leucine dehydrogenase. The 2-ml final reaction mixture contained 100 mM glycine/NaCl/NaOH buffer, pH 10.6, 50 mM valine, 0.5 mM NAD^+, and 0.1 ml of enzyme solution. The reaction was started by the addition of cell-free extract, and the mixture was incubated at 30°C. A control was performed under the same conditions without addition of valine. The increase in NADH was monitored at 340 nm. Enzyme activity is expressed as nmoles product formed per minute for each milligram of protein (nmol. min^{-1} mg^{-1}prot).

Ketoisovalerate dehydrogenase (KIVDH). The reaction mixture contained, in a final volume of 0.75 ml: phosphate buffer, pH 7.0, 100 mM; $MgCl_2$, 1.33 mM; NAD^+, 8 mM; cysteine hydrochloride, 4 mM; thiamine pyrophosphate, 0.8 mM; HS-CoA, 0.24 mM; ketoisovalerate, 4 mM. The reaction was started by adding enzyme extract, allowed to continue for 10 min at 25°C, and stopped by addition of trichloroacetic acid (TCA; 10%, wt/vol). After centrifugation, the isobutyryl-CoA produced was measured in the supernatant fluid by HPLC. A control was performed under the same conditions by adding TCA before the enzyme. Enzyme activities are expressed as nmoles of isobutyryl-CoA formed per minute for each milligram of protein (nmol · min^{-1} · mg^{-1}prot).

High-performance liquid-chromatography (HPLC) analysis. Isobutyryl-CoA in the assay mixture was detected by HPLC, measuring UV absorption at 255 nm. The equipment comprised a multisolvent delivery system (Spectraphysics SP8800 model), U6K injector, variable wavelength detector (Spectra Focus system), personal computer (IBM), and C18 column (Colosil, 6 mm × 15 cm, 5 mm particle diameter). The sample volume was 25 µl. Separation was achieved with a linear gradient from 0 to 30% methanol in acetate buffer, 50 mM, pH 5.3, for 40 min, at a flow rate of 2 ml · min^{-1}. For spiramycin estimation a C18 column was used (Novapak; 3.9 × 150 mm; 5 mm particle diameter). The analysis was done in isocratic conditions in the presence of a mixture of acetonitrile (20%) and 1% sulfuric acid (80%). After injection of the sample (20 µl), the analysis was started at 1 ml · min^{-1}. Before each injection, the column was equilibrated for 20 min with the same mixture. Spiramycin was detected by measuring absorbance at 238 nm.

Chemicals. Spiramycin was supplied by Rhône-Poulenc Santé (France); other reagents were obtained from Sigma and Fluka.

Results

Kinetics of growth and spiramycin production with two sources of carbon. Cultures of *S. ambofaciens* were grown in basal medium with either dextrins or glycerol (30 g · L^{-1}) as sole carbon source and valine (50 mM) as the nitrogen source (Fig. 1). *S. ambofaciens* grew faster on glycerol (μ_{max} = 0.07 h^{-1}) than on dextrins (μ_{max} = 0.03 h^{-1}). The final biomass obtained with dextrins and glycerol was approximately similar (5 g · L^{-1}). Glycerol was consumed at a higher specific rate (q_{gly} = 0.1 g · h^{-1} · g^{-1}CDW) than dextrins (q_{dex} = 0.04 g · h^{-1} · g^{-1}CDW). Spiramycin production was better with dextrins. In both cases, spiramycin was detected at 72 h fermentation and produced with a maximum specific production rate (q_{sp}) of 0.35 and 0.05 mg · h^{-1} · g^{-1} CDW respectively on dextrins and glycerol. With glycerol, the final volumetric spiramycin production was approximately 80% less than that obtained with dextrins. The intracellular ATP content measured on dextrins, at the beginning of spiramycin production (72 h), was 60% less than on glycerol.

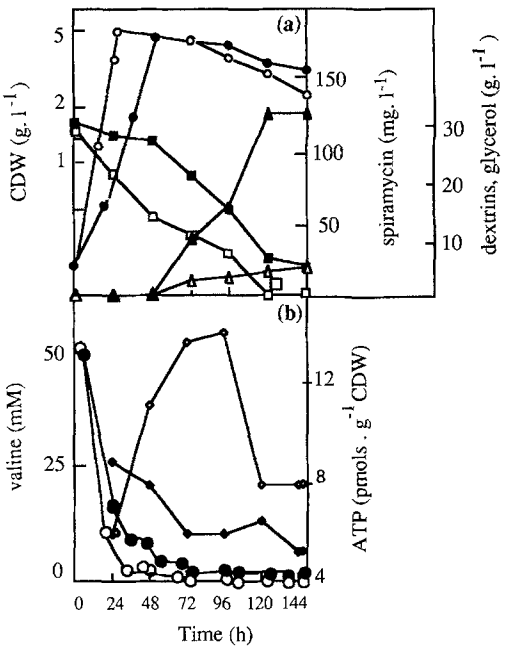

Fig. 1. Effect of dextrins (closed symbols) and glycerol (open symbols) on growth (●, ○), spiramycin production (▲, △), carbon source consumption (■, □) (**a**), valine consumption (●, ○), and intracellular ATP content (◆, ◇) (**b**).

Effect of increasing initial glycerol concentrations on growth and spiramycin production. Several fermentations with valine, 50 mM, as nitrogen source and increased concentrations of glycerol (5, 10, 20, 30, 40, 60 and 100 g · L^{-1}) as carbon source were carried out.

Increasing glycerol up to 20 g · L^{-1} caused an increase in the specific growth rate and a diminution in the pH. The final biomass continued to increase until 30 g · L^{-1} and then remained constant. From this

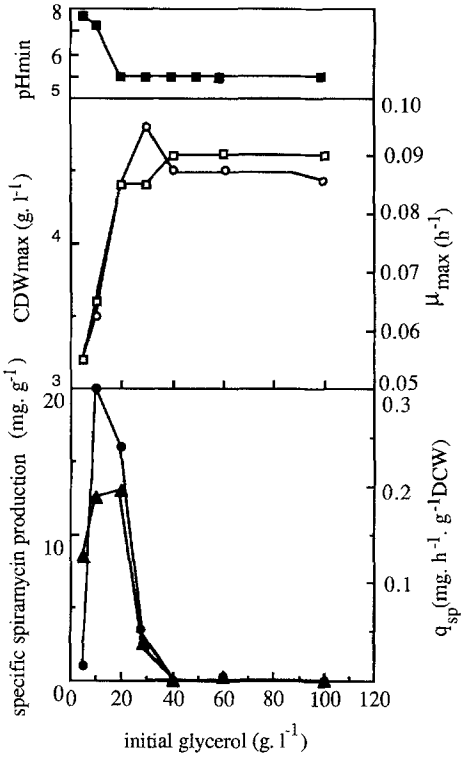

Fig. 2. Effect of increased initial concentrations of glycerol on pH (■), cell dry weight (CDW) (○), specific growth rate (□), specific spiramycin production (▲), and specific spiramycin production rate (●).

concentration and above, glycerol was no longer a growth-limiting factor for final biomass. Specific spiramycin production (13 mg · g^{-1}CDW) and q_{sp} (0.3 mg · h^{-1} · g^{-1}CDW) were maximum at 10 g · L^{-1} glycerol. Increasing glycerol up to 40 g · L^{-1} suppressed spiramycin biosynthesis (Fig. 2).

In order to make sure that this effect did not result from pH decrease or oxygen limitation, a fermentation on media containing 30 g · L^{-1} of glycerol and 50 mM valine was carried out in a fermentor (2 L) with control of the pH at 7.0 ± 0.1 and the oxygen at 25% ± 5% of saturation. The production remained lower than that obtained with 10 g · L^{-1} glycerol and 50 mM of valine. It was about 4 mg · g^{-1} CDW (results not shown).

Effect of glycerol addition, at 56 h, on spiramycin production. In order to prevent the stimulatory effect of rapidly metabolized carbon source on the specific growth rate, different concentrations of glycerol (5, 10, 20, 30, 40 and 60 g · L^{-1}) were added, on the basal medium with dextrins 15 g · L^{-1} and valine 25 mM, at the end of exponential phase under conditions of nitrogen limitation (56 h).

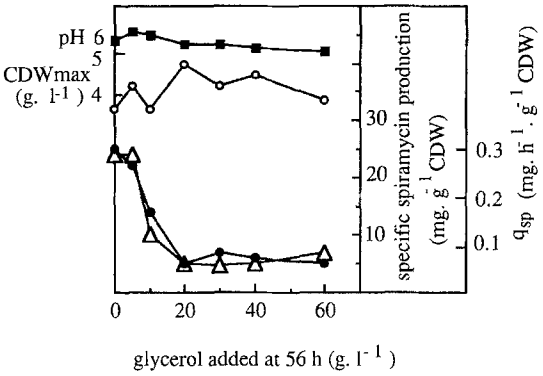

Fig. 3. Effect of glycerol concentrations, added at 56 h, on pH (■), cell dry weight (○), specific spiramycin production (△), and specific spiramycin production rate (●) with the basal medium with valine 25 mM and dextrins 15 g · L^{-1}.

Addition of glycerol did not increase either μ_{max} or CDW_{max}. In these conditions the carbon source was no longer a growth-limiting factor, but valine probably was. Meanwhile, the biomass was maintained at a high level of approximately 4.5 g · L^{-1}. The pH did not vary significantly. Increasing glycerol concentration up to 20 g · L^{-1} decreased the specific spiramycin production and the specific spiramycin production rate by 80% (Fig. 3).

Determination of the nature of glycerol effect

Comparison of glycerol or streptomycin time addition on spiramycin production. In order to begin to elucidate the mechanism implicated in the negative glycerol effect on spiramycin biosynthesis, we attempted to locate the phase of spiramycin enzyme synthesis. The effect on spiramycin production of streptomycin (an inhibitor of protein biosynthesis) and glycerol additions, at different incubation times, were studied using the basal medium with dextrins 30 g · L^{-1} and valine 50 mM (Table 1).

Addition of 100 mg · L^{-1} of streptomycin to cultures before the onset of antibiotic biosynthesis (48 h) suppressed spiramycin production. Addition of streptomycin at the onset of spiramycin production (72 h) exerted a strong but not total negative effect (reduction of 80% of specific spiramycin production). Addition of streptomycin after the onset of production had no significant effect on spiramycin production when compared with the control.

Addition of glycerol (60 g · L^{-1}), in the same conditions, showed approximately the same effect on spiramycin production as streptomycin. However, when glycerol was added after the onset of the production, the specific spiramycin production was reduced by 45%.

Table 1. Effect of streptomycin (100 mg · L^{-1}) or glycerol (60 g · L^{-1}) addition at different incubation times on spiramycin production by use of basal medium with valine 50 mM and dextrins 30 g · L^{-1}

Time addition of streptomycin or glycerol	Specific spiramycin production (mg · g^{-1} DCW)	
	Streptomycin (100 mg · L^{-1})	Glycerol (60 g · L^{-1})
Before the start of spiramycin production		
48 h	0	3
At the start of spiramycin production		
72 h	5	8
After the start of spiramycin production		
96 h	26	14
120 h	23	
Control	25	

Table 2. Effect of streptomycin or glycerol additions on spiramycin production with RCS (resting cell systems)

Conditions of RCS[a]	Age of culture		
	48 h	72 h	96 h
	Maximum specific spiramycin production (mg · g^{-1} DCW)		
MWN	10	9.5	0
$MWNS^+$	17.5	14.5	0
$MWNG^+$	0	0	0
$MWNS^+G^+$	0	0	0

[a] MWN, the basal medium without nitrogen containing dextrins 30 g · L^{-1} as carbon source; $MWNS^+$, the basal medium without nitrogen containing dextrins 30 g · L^{-1} as carbon source with streptomycin 100 mg · L^{-1} addition; $MWNG^+$, the basal medium without nitrogen containing dextrins 30 g · L^{-1} as carbon source with glycerol 60 g · L^{-1} addition; $MWNS^+F^+$, the basal medium without nitrogen containing dextrins 30 g · L^{-1} as carbon source with simultaneous addition of Streptomycin (100 mg · L^{-1}) and glycerol (60 g · L^{-1}).

According to the results above, glycerol seemed to exert its negative effect all through the duration of the fermentation, although the effect was less intense after the onset of spiramycin production. Glycerol appeared to have both inhibitory and repressive actions on spiramycin biosynthesis. To prevent the eventual negative influence of glycerol on spiramycin biosynthesis via increase of the specific growth rate and to confirm the nature of the negative effect of glycerol, we did the next experiment.

Suppression of spiramycin production by glycerol in resting cell system (RCS). Cultures of *S. ambofaciens* were carried out on medium that favored spiramycin production (dextrins 30 g · L^{-1} and valine 50 mM). The cells were harvested at different incubation times (48 h "before the onset of spiramycin production"; 72 h "at the onset of spiramycin production"; and 96 h "after the onset of spiramycin production," and resuspended in the RCS medium without nitrogen, without (MWN), or with addition of streptomycin 100 mg · L^{-1} ($MWNS^+$), with glycerol 60 g · L^{-1} ($MWNG^+$) or with glycerol 60 g · L^{-1} plus streptomycin 100 mg · L^{-1} ($MWNG^+S^+$). Table 2 recapitulated the results obtained.

In the systems MWN and $MWNS^+$, spiramycin was produced in the cells harvested at 48 and 72 h with specific spiramycin production, which was not significantly different according to the time of harvest. The cells of 96 h were not productive. These results indicated that the presence of enzymatic systems necessary for spiramycin biosynthesis were set up before 48 h. These enzymatic systems are not functional after 96 h of incubation. The cells harvested at 48 and 72 h produced more spiramycin in the presence of streptomycin than in its absence (increases of 75% and 50% of the specific spiramycin production at 48 and 72 h respectively). The stimulatory streptomycin effect on spiramycin biosynthesis could suggest the reorientation of metabolites from protein biosynthesis to antibiotic production.

Whatever the age of the cells used (48 or 72 h), the addition of glycerol in the presence or absence of streptomycin suppressed spiramycin production. The inhibitory action of the glycerol on spiramycin production was confirmed.

Besides the inhibitory effect of glycerol on spiramycin biosynthesis, its eventual repressive action had been examined. Indeed, since it was demonstrated in previous study [11] that valine, in the presence of glycerol, served as nitrogen, carbon, and energy sources, the regulation by glycerol of two enzymes of valine catabolism, VDH and KIVDH, suppliers of spiramycin aglycone precursors, was examined in the next experiment.

Kinetics of VDH and KIVDH biosynthesis in the presence of dextrins and valine. VDH and KIVDH specific activities were measured in fermentation with the basal medium described above with 50 mM valine and 30 g · L^{-1} dextrins (control fermentation) (Fig. 4).

Spiramycin was produced with a specific spiramycin production rate of 0.35 mg · h^{-1} · g^{-1} CDW. Valine was consumed with a specific uptake rate of 0.8

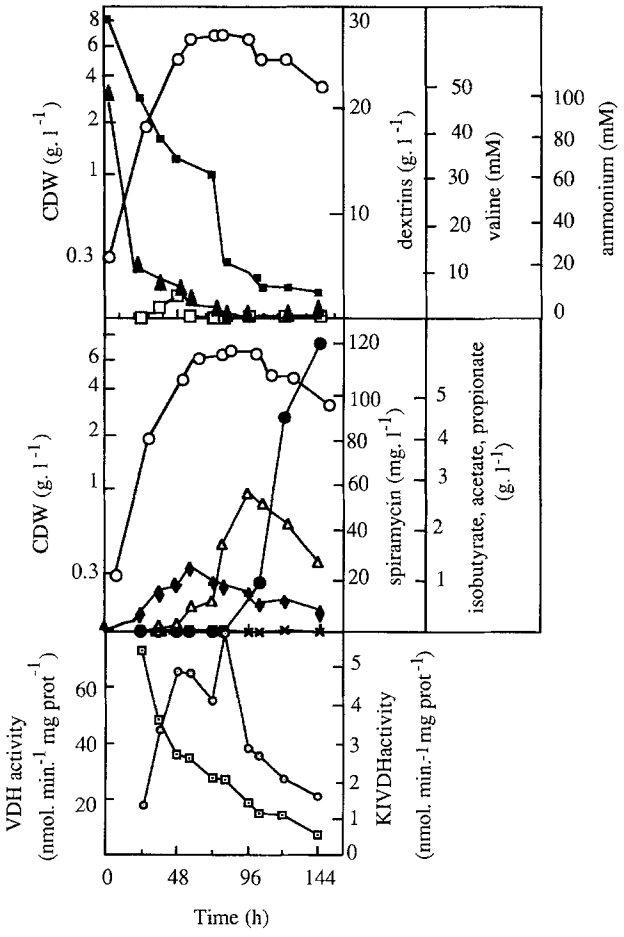

Fig. 4. Kinetics of growth (○), dextrins consumption (■), valine consumption (▲), ammonium concentration (□), spiramycin production (●), acetate (◆), isobutyrate (△) and propionate (x) evolution, VDH (⊡) and KIVDH (⊙) specific activities on the basal medium with dextrins 30 g · L^{-1} and valine 50 mM.

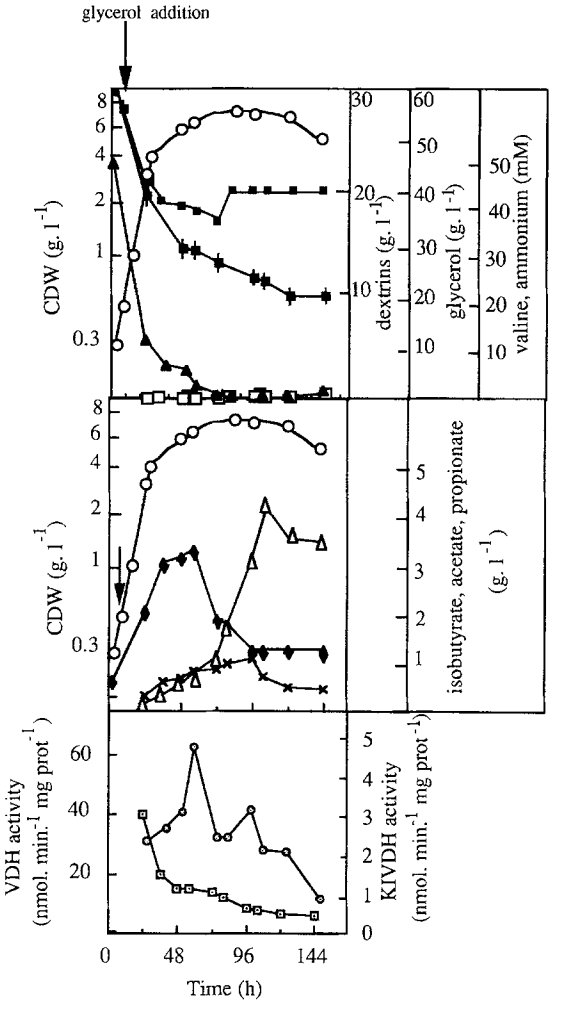

Fig. 5. Kinetics of growth (○), dextrins consumption (■), glycerol consumption (▮), valine consumption (▲), ammonium (□), acetate (◆), isobutyrate (△), and propionate (x) evolution, VDH (⊡) and KIVDH (⊙) specific activities on the basal medium containing dextrins 30 g · L^{-1} and valine 50 mM with a pulse of glycerol 60 g · L^{-1} at 12 h fermentation.

mmol · h^{-1} · g^{-1}CDW. Production of 1.2 g · L^{-1} acetate and 3 g · L^{-1} isobutyrate, observed respectively during exponential and stationary phases, were reassimilated during intensive phase of spiramycin production. Isobutyrate was excreted with a specific rate of about 0.15 mmol · h^{-1} · g^{-1}CDW and with a yield ($Y_{isobutyrate/valine}$) of 0.6 mol · mol^{-1}. Traces of propionate were also detected.

The specific activity of VDH was relatively high at 24 h incubation (78 nmol · min^{-1} · mg^{-1}prot) and decreased (60% in 2 days) as soon as the growth slowed down. During the growth phase, KIVDH specific activity reached a maximum of 5 nmol · min^{-1} · mg^{-1} prot at 48 h. This KIVDH activity decreased, then showed at the stationary phase (80 h) a second maximum specific activity of 6 nmol · min^{-1} · mg^{-1} prot.

Effect of glycerol pulse on growth, spiramycin, VDH, and KIVDH biosynthesis. Glycerol effect on growth, spiramycin production, VDH and KIVDH specific activities was determined in a fermentation which consisted of glycerol pulse (60 g · L^{-1}) at 12 h incubation on a culture begun on a basal medium with dextrins (30 g · L^{-1}) and valine (50 mM) (Fig. 5).

The pulse of glycerol caused an increase of the growth (μ_{max} = 0.06 h^{-1}). Specific valine consumption rate was reduced by 50% (q_{val} = 0.4 mmol · h^{-1} · g^{-1}CDW). Glycerol and dextrins were consumed with a specific uptake rate of 0.1 g^{-1} · h^{-1} · g^{-1} CDW. During active growth phase, both dextrins and glycerol were consumed simultaneously. Afterwards, glycerol was mostly assimilated, whereas dex-

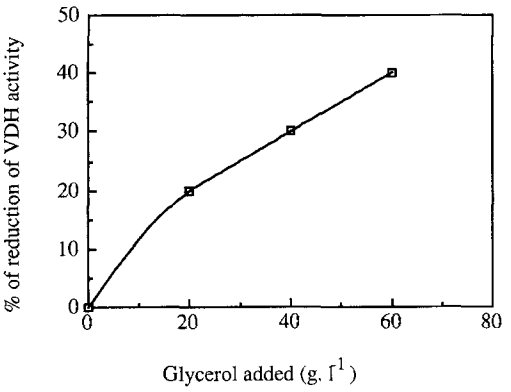

Fig. 6. Effect on VDH specific activity of different concentrations of glycerol, added at 12 h fermentation, on the basal medium with dextrins 30 g · L^{-1} and valine 50 mM.

trins were not. Spiramycin biosynthesis was suppressed. Glycerol pulse provoked the excretion of 3 g · L^{-1} of acetate and 1 g · L^{-1} of propionate, which were markedly reconsumed. In contrast, isobutyrate was produced with a specific rate of 0.07 mmol · h^{-1} · g^{-1}CDW and a yield ($Y_{isobutyrate/valine}$) of 1 mol · mol^{-1}, but it was not significantly reassimilated.

VDH specific activity showed the same evolution than on dextrins as the sole carbon source. But its maximum activity was reduced by 40% by the pulse of glycerol in comparison with the control. This drop was well correlated with the concentration of glycerol pulse: drops of 19, 28, and 40% were obtained with 20, 40 and 60 g · L^{-1} glycerol pulses respectively (Fig. 6).

KIVDH activity followed the same pattern as dextrins as unique carbon source; however, the maximum specific activity detected at the stationary phase (96 h) was reduced by 50% (3 nmol · min^{-1} · mg^{-1}prot) in the case of glycerol pulse (Fig. 5).

Discussion

It has been shown in several systems that production of antibiotics and secondary metabolites was affected negatively when a rapidly used carbon source was utilized for growth [14]. *S. ambofaciens* was found to grow on a variety of carbon sources including dextrins and glycerol. In the presence of dextrins as slowly assimilated carbon and energy source, spiramycin production was higher than with glycerol. The low spiramycin production with glycerol could be attributed to two actions: one, glycerol probably generated conditions of excess energy. This view was supported by the intracellular ATP content of the cells, which was lower in the presence of dextrins than with glycerol. Two, glycerol led to a high specific growth rate. This fact was confirmed by the use of increased initial concentrations of glycerol. Indeed, increased concentrations of glycerol provoked a decrease of antibiotic biosynthesis which was parallel to an increase in specific growth rate. By controlling the pH and aeration in a fermentor, we proved that the inhibitory effect was not due to changes in the pH of the culture nor to the depletion of O_2 because the effect occurred at low biomass concentration where oxygen could not have been limiting.

The time of addition of glycerol seemed to have an important effect on spiramycin production. If glycerol was added prior to the onset of spiramycin synthesis, severe reduction of antibiotic synthesis occurred. If glycerol was added after the onset of production, the drop of spiramycin production was less intense.

Addition of different concentrations of glycerol at the end of the exponential phase (conditions of nitrogen limitation) showed that the inhibitory action of this rapidly metabolized carbon source was not due only to the stimulatory effect on the specific growth rate.

Our experiments using streptomycin (an inhibitor of protein synthesis) in medium allowing spiramycin production and in a resting cell system showed that the enzymatic systems responsible for the secondary metabolism are set up generally at the end of the exponential growth phase. At the stationary phase, they are totally inactivated. Glycerol seemed to exert both inhibitory and repressive actions on the enzymatic machinery of spiramycin biosynthesis.

Analogous experiment with addition of glycerol in a producer culture medium and in resting cell systems showed that, contrary to the glycerol effect observed in other system, glycerol acted as an inhibitor of gene products already expressed (secondary metabolism enzymes). Wei-Shou and associates [21] described a resting cell system in *S. clavuligerus* where simultaneous addition of glycerol and an inhibitor of protein synthesis allowed cephalosporin production when this biosynthesis did not occur with glycerol added alone. Their results suggested that the negative effect of carbon source was due to formation of an enzyme inactivating one or more of the cephem synthetases. In our case, glycerol did not seem to act by the same mechanism. The inhibitory effect of rapidly assimilated carbohydrates on antibiotic synthesis was reported in *S. clavuligerus* [1].

In this study, based on our previous results [11] which reported that valine, in the presence of glycerol, served as nitrogen, carbon and energy sources, the regulation by glycerol of two enzymes of valine

catabolism, VDH and KIVDH, generator of aglycone spiramycin precursors, was examined. We showed that glycerol partially repressed VDH and KIVDH. The pulse of glycerol provoked a sharp decrease in the specific valine uptake. Indeed, glycerol pulse was found to reduce VDH activity (40%). KIVDH activity in the growth phase was not affected; however, the specific activity detected at the stationary stage was reduced (50%). With the partial repression of VDH and KIVDH, accumulation of isobutyrate was observed. $Y_{isobutyrate/valine}$ equal to 1 indicated that glycerol prevented catabolism of isobutyryl-CoA. Glycerol seemed to exert its regulatory negative effect downstream of isobutyrate, repressing the systems of activation or assimilation of isobutyrate. Isobutyryl-CoA dehydrogenase could constitute one of the enzymatic targets of glycerol effect. Glycerol probably had other sites of action in secondary metabolism. Previous studies reported that the regulatory mechanism by glycerol of cephamycin biosynthesis in *C. acremonium*, *S. clavuligerus*, and *S. lactamdurans* was due in part to the repression and inhibition of α-aminoadipyl-cysteinyl-valine sythetase, cyclase, epimerase, and expandase [3, 9].

In addition, glycerol could repress genes involved in spiramycin biosynthesis. Thus, our first protein analysis, on polyacrylamide gel electrophoresis in a gradient of concentrations, showed that in the culture where the spiramycin production was prevented by the pulse of glycerol, the profile looked different: some proteins bands were absent (results not shown).

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