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Effect of hydroxylic solvents on cell growth, sporulation, and esterase production of *Bacillus licheniformis* S-86

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Abstract

Thermoresistant and alcohol-tolerant bacterium *Bacillus licheniformis* S-86 can produce a stable esterase in high organic solvents concentrations. In this work, the effects of seven alkanols on cell growth, sporulation, and esterase production of *Bacillus licheniformis* S-86 were investigated. A reciprocal relationship between maximal organic solvent concentrations tested with log *P* was observed but there was no correlation between the alcohol log *P* and the esterase production. Esterase specific activity was approximately two-fold higher in cultures supplemented with C3–C5 alkanols than the control. The addition of 3-methylbutan-1-ol to the culture medium, strongly depleted sporulation compared to the control by 10^4-fold CFU/ml; but, the esterase production was enhanced more than two times under the same conditions.
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1. Introduction

During the last three decades, the use of enzymes and whole cells catalysts for resolution of racemates and synthesis of chiral and enantiopure compounds performed in organic solvent have attracted considerable attention in biotechnology [1]. Esterases (triacylglycerol lipases and carboxylesterases) were successfully used in transesterification reactions, which include the resolution of racemic mixtures, enantio- and regio-selective hydrolysis and synthesis of natural and non-natural drugs, detergents, polymers, and additives with industrial relevance [1,2]. Also, aminolysis and ammonolysis reactions were carried out by lipases in organic solvents [3]. However, most reports describing the use of microbial esterases were restricted to commercially available sources, from fungi (*Rhizomucor miehi, Rhizopus niveus,* and *Rhizopus orizae*), yeasts (*Candida antarctica*, *Candida cylindracea*, and *Candida rugosa*) and some Gram-negative bacteria, such as *Achromobacter*, *Alcaligenes*, *Chromobacterium*, and *Pseudomomas* species [4–8]. Therefore, some limitations on esterases reactions, such as low reaction rates, low product yields and high enzyme deactivation rates in organic solvents were reported [9,10].

Screening, isolation, and identification of novel microbial strains able to tolerate and survive in the presence of toxic organic solvent concentrations were underdeveloped until the last 5 years. This was because of the difficulties of maintaining cell viability on highly toxic organic solvent environment and as a result of the anthropomorphic view of microbial life (e.g. aqueous media, 37 °C, pH 7.0). Also, organic solvent-tolerant micro-organism, and viable cells for enzyme production in extreme environments, such as organic solvents, have received little attention, but is now growing up as a new area of extremophiles.

Solvent toxicity was rationalized considering the cellular membrane as the main organic solvent target by the Hansch parameter or log *P*, which is defined as the logarithm of the partition coefficient of solvent in octanol–water phase system [11,12]. Organic solvents with log *P* between approximately 1 and 5 are considered extremely toxic for micro-organisms [12,13]. Nevertheless, the limits of solvent toxicity to the cells apparently are not strict and depend not

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only on strains and species assayed, but also of experimental conditions (e.g. medium, pH, temperature, ionic strength, inoculums).

Effects of non-polar organic solvents particularly in Gram-negative bacteria were studied in great detail [14–17]. In contrast, studies developed using polar organic solvents, like alkanols excluding ethanol, in Gram-positive micro-organisms are very scarce. Probably, because short-chain aliphatic alcohols have been used for a long time as disinfectants, preservatives, and microbiocidal compounds. Also, high-enzyme producer Gram-positive micro-organisms were underestimated in relation to organic solvent tolerance. Furthermore, some reports have shown that wild-type organic solvent-tolerant micro-organisms are able to produce enzymes with high stability in the presence of non-aqueous solvents [13,18,19]. In fact, an alcohol-tolerant wild-type *Bacillus licheniformis* S-86 isolated in this laboratory was able to produce a stable esterase in organic solvents [20]. In the present work, the effect of alkanols on cell growth, sporulation, and enzyme production of *B. licheniformis* S-86 was studied.

2. Materials and methods

2.1. Micro-organism and media

The thermoresistant and alcohol-tolerant *B. licheniformis* S-86 strain, isolated from soil at 55 °C, was used. In order to prepare the inocula, *B. licheniformis* S-86 was grown overnight on a tributyrin agar plate at 55 °C. Cells were then picked from the agar plate and pre-cultured in the enzyme production (EP) medium for 3 h at 50 °C (exponential phase of growth). Those cells were then used to inoculate fresh EP media with starting absorbance at 560 nm (A_{560}) between 0.05 and 0.10, where growth and enzyme production experiments were carried out. The tributyrin agar contained (g/l): peptone, 5.0; yeast extract, 3.0; tributyrin, 10.0; and agar 15.0. The enzyme production (EP) medium contained (g/l): $NaNO_3$, 1.2; KH_2PO_4, 3.0; K_2HPO_4, 6.0; $MgSO_4{\cdot}7H_2O$, 0.2; $CaCl_2$, 0.05; $MnSO_4{\cdot}H_2O$, 0.01; $ZnSO_4{\cdot}7H_2O$, 0.001; peptone, 2.50; yeast extract, 1.50; and maltose, 10.0.

Spore counts were assayed on nutrient–agar containing (g/l): peptone, 5.0; beef extract, 3.0; NaCl, 8.0; and agar, 15.0.

2.2. Growth and enzyme production experiments

To study the effects of alkanols on cell growth and esterase production of *B. licheniformis* S-86, cells were grown in Erlenmeyer flasks (125 ml) containing 15 ml of EP media at 50 °C supplemented or not with organic solvent (ethanol (4%); propan-2-ol (3%); butan-1-ol (1%); 2,3-butanediol (6%); 3-methylbutan-1-ol (0.4%); hexan-1-ol (0.1%); and heptan-1-ol (0.05%)) for 48 h on a rotatory shaker at 3.3 Hz. Growth was measured as a function of optical density at 560 nm (A_{560}) in a spectrophotometer (Metrolab 1250, Corswant, Argentina). Each solvent was tested in the maximal concentration that allowed the growth above 1 unit of A_{560} the first 24 h and did not affect the esterase production; when the concentration of the solvent was higher than the used for each one, caused the growth inhibition of the micro-organism or the enzyme production decrease.

2.3. Enzyme assays

Cell-free enzyme activities were assayed in the supernatant by centrifuging the cultures at 8000 × *g* for 10 min (4 °C). Alternatively, esterase activity was assayed in partially purified extracts obtained by precipitation of crude extract with cold acetone (−20 °C) at 0 °C for 6 h. The precipitate was dried, weighed, and concentrated 10 times of the original volume using 50 mM Tris–HCl buffer (pH = 7.0).

Esterase activity was quantified using the *p*-nitrophenyl derivative of acetate. The reaction mixture contained 60 μl of 200 mM Tris–HCl buffer (pH 7.0), 5 μl of 74 mM *p*-nitrophenyl derivative, 305 μl of water, and 30 μl of enzyme. Enzyme reactions were carried out at 37 °C for 30 min, and the production of *p*-nitrophenol was determined spectrophotometrically at 400 nm in a 1-cm light path (Metrolab 1250, R. Corswant, Argentina). One enzyme unit (EU) was defined as the amount of enzyme producing 1 μmol of *p*-nitrophenol per minute.

2.4. Determination of protein concentration

Coomassie brilliant blue G-250 reagent was used to determine samples protein content using bovine serum albumin (Fraction V) as standard [21].

2.5. Spore counts

Spore counts were determined from cultures supplemented with 0.4% 3-methylbutan-1-ol and without solvent, in cell suspensions heated at 80 °C for 15 min. Samples were then diluted appropriately in 0.1% Tween 20 and aliquots were spread on nutrient-agar plates which were counted after aerobic incubation at 55 °C for 18 h.

2.6. Statistical treatment

All experiments were carried out in duplicate ($n = 2$). The error was calculated using the standard deviation.

3. Results and discussion

B. licheniformis S-86 isolated from a South American soil sample on tributyrin-supplemented medium was able to

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