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Selection of an extracellular esterase-producing microorganism

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SUMMARY

The conventional esterase plate-screening technique has been modified in order to obtain a better differentiation of high producing strains. A short exposure to Lugol's iodine solution after colony growth enhanced the contrast between the precipitation halo and the background. Batch cultures of a selected strain characterized as belonging to the *Bacillus subtilis* group showed a high level of extracellular activity at pH 6.6 to 8.0 and 35 °C. In crude extracts the optimum enzymatic activity was obtained at 35 °C and pH 7.0.

INTRODUCTION

In recent years there has been great interest in esterase-producing microorganism for the development of large-scale esterification processes for the synthesis of high value products from inexpensive raw materials [5,6,8]. One of the most promising applications are the synthesis of flavors and fragances for foods, cosmetics and perfumes [3].

The aim of the present study was to improve the standard esterase-screening technique, to characterize the bacteria producing high levels of extracellular enzyme, and to see what factors effect enzyme yield.

MATERIALS AND METHODS

Cultivation of bacteria

Defined salts medium was used to isolate strains. The medium contained (g/l): $(NH_4)_2SO_4$, 5.0; K_2HPO_4, 6.0; KH_2PO_4, 3.0; $ZnSO_4 \cdot 7H_2O$, 0.001; $MgSO_4 \cdot 7H_2O$, 0.010; $MnSO_4 \cdot 4H_2O$, 0.001; $CaCl_2 \cdot 2H_2O$, 0.050; $FeSO_4 \cdot 7H_2O$, 0.001; sodium citrate, 1.00 and soluble starch, 10.0. The pH was adjusted to 7.0 with NH_4OH prior to sterilization. Purification and maintenance procedures used for spore-forming bacteria were performed according to methods previously described [9].

Growth experiments were carried out in an MTS medium containing, in g per liter: starch, 1.5; meat extract, 5.0, tryptone, 17.5 (pH 7.5). The selected strain was grown in Erlenmeyer flasks (250 ml) containing 50 ml of medium at 37 °C overnight on a rotatory shaker at approx. 180 rpm. All experiments were carried out in duplicate. Growth was measured as a function of optical density at 600 nm in a spectrophotometer (Bausch & Lomb 20).

Screening of microorganism

Esterase-producing colonies were detected by their ability to hydrolyse Tween 20. Petri dishes containing 20 ml of the following medium were used (g/l): meat extract, 3.0; peptone, 5.0; polyoxyethylene-sorbitan monolaurate (Tween 20), 5.0; $CaCl_2 \cdot 2H_20$, 0.4; agar, 15.0. The plates were incubated at 45 °C for 48 h. Esterase-producing colonies were surrounded by a white precipitate of calcium laurate. In order to enhance the contrast with the background, 5.0 ml of Lugol's iodine solution (KI, 20.0; I_2, 2.0) was added to each plate and removed after 1 min of exposure.

Enzyme assay

The esterase production obtained in growth experiments with MTS medium was assayed by adding 100 μl of filtrate samples (0.22 μm pore size) to 200 μl of 500 μg/ml α-naphthylacetate dissolved in an ethanol/water mixture (1:2). The mixture was incubated at 35 °C for 30 min. The reaction was stopped by the addition of 50 μl of 4.0 M urea and made alkaline by adding 50 mM borax/40% ethanol (v/v) solution made up to a total volume of 2.80 ml.

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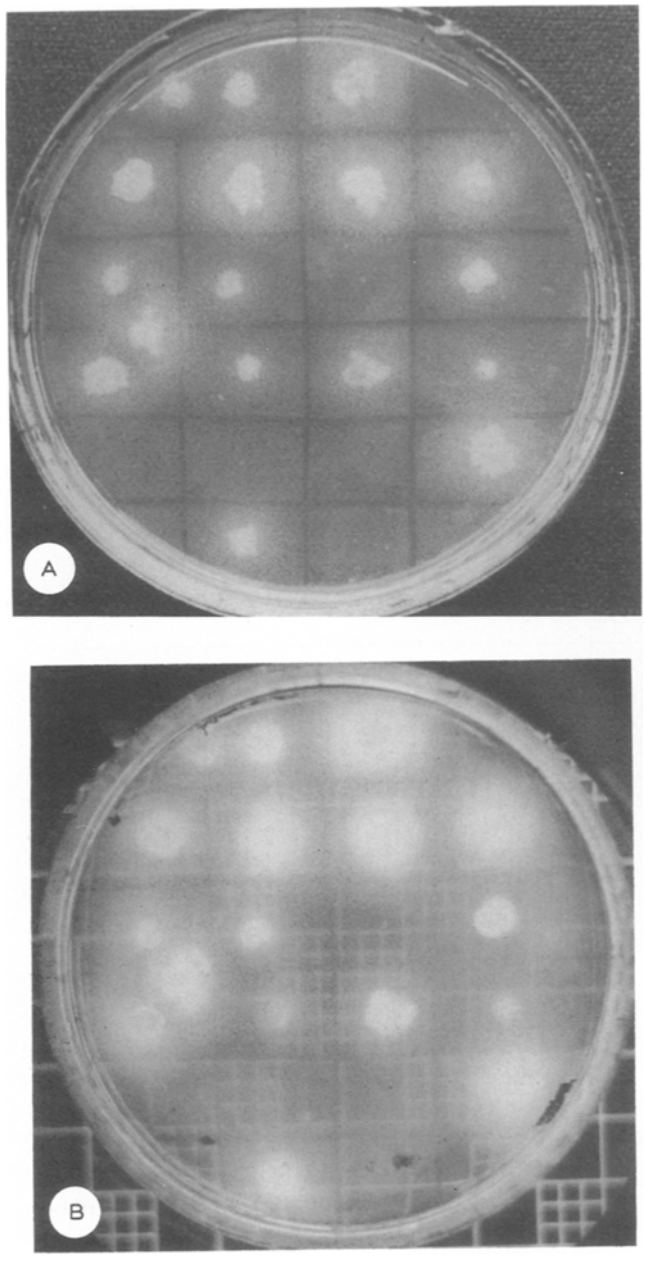

Fig. 1. Screening of esterase-producing microorganism. Classical method (A) and new method (B).

Then 200 μl of an aqueous solution of Fast blue (1.0 mg/ml) was added and the sample was left for 10 min at room temperature. The resulting color was measured spectrophotometrically at 515 nm (Jasco 7850). Appropriate blanks and standards were measured simultaneously. An enzymatic unit (EU) was defined as the quantity of enzyme needed to produce 1 μmol/ml of α-naphthol per min under our assay conditions.

Microbial identification

The selected strain *B. subtilis* MIR 16 was characterized morphologically and biochemically according to methods previously described [2,7,9].

pH and temperature optima

For determination of pH optimum, samples were incubated with the substrate in the following 50 mM buffers: acetate pH 5.0 to 5.4, sodium phosphate in the 5.8 to 7.0 pH range and borate pH 8.0 to 8.4 at 35 °C. To determine the optimum temperature, the assay mixtures were incubated for 30 min at pH 7.0 at temperatures varying from 25 to 60 °C and the esterase activity determined as described in this paper.

RESULTS AND DISCUSSION

Preliminary screen

Different strains (280) from our collection belonging to the genus *Bacillus*, isolated in the course of previous screenings for thermoresistant amylolytic bacteria, were tested for their ability to produce esterases. One hundred strains which produced colonies with a mean diameter of 4–6 mm on minimal medium after 24 to 48 h were selected. The procedure was repeated for confirmation, and 15 strains, which had a distinct precipitation halo with a diameter twice that of the colony diameter or more, were selected for further testing.

Further selection of the best esterase production under the assay conditions was not possible because of the undefined limits of the precipitation halos in the plate (Fig. 1A). Attempts to increase the definition by increasing the Tween concentration resulted in a decrease in colony size and alterations of the colony morphology. On the other hand, plates exposed to Lugol's iodine solution for a short time showed a yellow-brown colored background which contrasted sharply with the white precipitation halo (Fig. 1B). The resolution of the screening technique was, therefore, increased (see Fig. 1). This result could be due to the formation of a complex between $(I_3)^{1-}$ and free Ca^{2+}. The strain MIR 16 exhibited the largest halo out of all the strains examined, due to esterase production on solid medium. This was also demonstrated in liquid medium (data not shown).

Strain identification

On the basis of its physiological and morphological properties, the strain MIR 16 appeared to belong to the genus *Bacillus* and to the species *subtilis*. The selected strain MIR 16 had the following characteristics: Gram-positive, rod-shaped cells, cylindrical spore in subterminal position with no bulging. The strain was positive for the following tests: casein, dextran, gelatin, olive oil, pullulan,

starch and Tween 20; catalase assay, dihydroxyacetone production, methyl red test, nitrate reduction and Voges-Proskauer reaction. MIR 16 grew between pH 4.5–7.0 and at temperatures between 37–50 °C, and 2.0–5.0% NaCl. Substrates utilization includes: adonitol, esculin, amygdalin, L-arabinose, arbutin, cellobiose, D-fructose, galactose, β-gentiobiose, α-methyl-D-glucoside, D-glucose, glycerol, inositol, inulin, lactose, maltose, mannitol, D-mannose, D-raffinose, rhamnose, ribose, saccharose, salicin, sorbitol, trehalose, D-turanose and L-xylose.

It was negative for the following tests: Tween 80 and urea, anaerobic growth, citrate utilization, gas production from glucose, indole production and phenylalanine. Substrates not utilized include: D-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, L-fucose, gluconate, 2-keto-gluconate, 5-keto-gluconate, *N*-acetyl-glucosamine, glycogen, D-lyxose, α-methyl-D-mannoside, melezitose, L-sorbose, D-tagatose, xylitol and β-methyl-xyloside. No growth was observed in 7.0% NaCl, 60 °C or on MacConkey agar.

Enzyme production

Results of growth experiments are shown in Fig. 2. The extracellular esterase activity was closely related to growth. Different results were reported in previous work with *Bacillus subtilis* Marburg, whose esterase activity was detected at the onset of sporulation (late exponential phase) [4].

The esterase activity of MIR 16 reached a maximum of 14.0 EU/ml after 6 h of growth under our experimental conditions. This activity remained constant for at least 5 h (data not shown) without any observed cellular lysis. The esterase activity found in *B. subtilis* strain MIR 16 is much higher than that reported for *B. subtilis* Marburg strain although the growth medium is different [4].

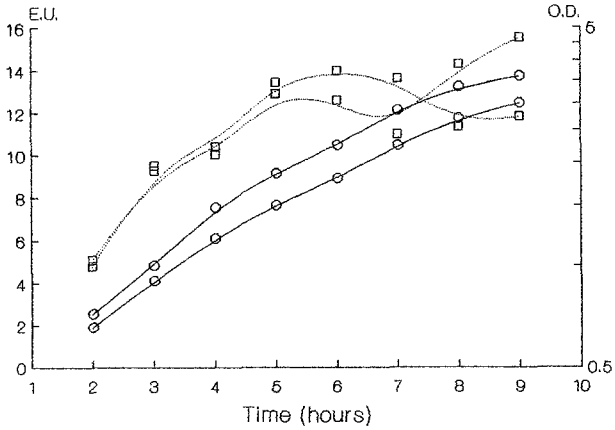

Fig. 2. Esterase activity (□) and bacterial growth (○) exhibited by *B. subtilis* MIR 16 at 37 °C. Optical density at 600 nm was determined as a measure of cell growth.

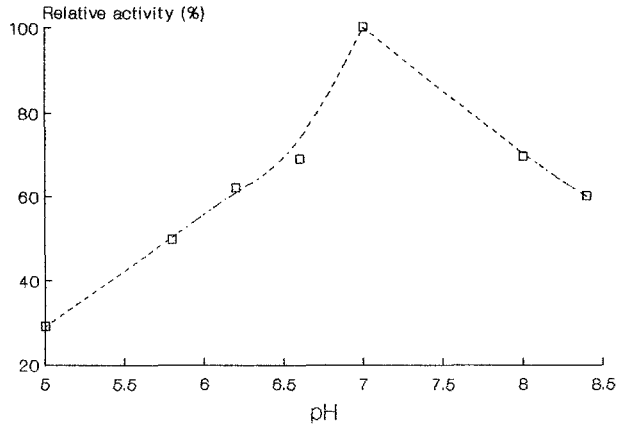

Fig. 3. Effect of pH on esterase activity. The enzyme activity was assayed at 35 °C for 30 min in 50 mM buffers: acetate (5.0 and 5.4 units), phosphate (5.8 to 7.0 pH range) and borate (8.0 to 8.4 units).

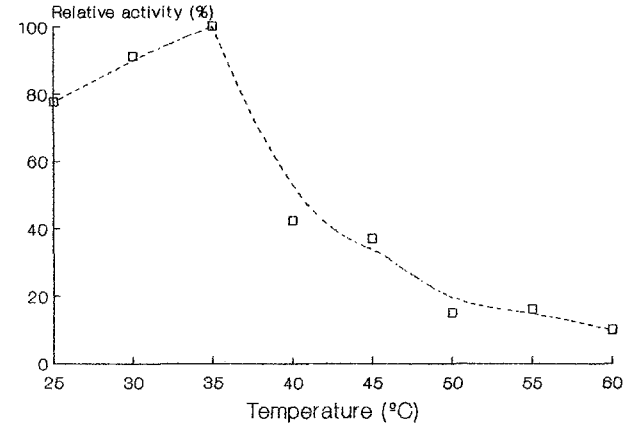

Fig. 4. Effect of temperature on esterase activity. The enzymatic activity was measured at pH 7.0 in 50 mM phosphate buffer for 30 min.

The high enzymatic activity found in liquid medium with *B. subtilis* MIR 16 confirms the value of the screening technique. The data about the effect of pH and temperature on the esterase activity are shown in Figs. 3 and 4, respectively. The optimal activity of the enzyme was found at pH 7.0. At pH 6.6 and 8.0, only 69% of the activity can be detected. The temperature optimum was 35 °C, and a slight increase in temperature showed rapid inactivation of the enzyme in the crude extract. Similar results were reported previously for intracellular esterases in *B. subtilis* Marburg [4] and *Nocardia mediterranei* [8].

The enzymatic activity measured with α-naphthyl propionate was very similar (data not shown). The enzyme could be tentatively considered an aryl-esterase: EC 3.1.1.2.

The major advantages of the screening technique de-

scribed in this paper are: (i) low and high esterase-producing strains can be detected on the same plate without any special equipment; and (ii) the assay is simple, inexpensive and well-suited for the screening of large numbers of samples. The strain *Bacillus subtilis* MIR 16, isolated by using this technique, shows an extracellular esterase activity which is 100-times greater than the activity previously reported in the literature [3]. The species *B. subtilis* lends itself to increases in enzyme production by either genetic manipulation and/or medium improvement [1]. Further studies need to be done to enhance optimal conditions for esterase production by MIR 16. In addition, characterization and purification of this esterase will enable us to compare its properties to know esterases.

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REFERENCES

1 Debavov, V.G. 1982. The industrial use of Bacilli. In: The Molecular Biology of the Bacilli (Dubnau, D. ed.), Vol. 1, pp. 331–370, Academic Press. New York.

2 Gordon, R.E. 1977. The genus *Bacillus*. In: Handbook of Microbiology (Laskin, A.I. and Lechevalier, H., eds.), pp. 319–336. CRC Press, Boca Raton, FL.

3 Iwai, M., S. Okomura and Y. Tsujisaka. 1980. Synthesis of terpene alcohol esters by lipase. Agric. Biol. Chem. 44: 2731–2732.

4 Mamas, S. and J. Millet. 1975. Purification et propriétés estérase excrétée pendant la sporulation de *Bacillus subtilis*. Biochimie 57: 9–16.

5 Owusu, R.K. and D.A. Cowan. 1991. Isolation and partial characterization of a novel thermostable carboxylesterase from a thermophilic *Bacillus*. Enzyme Microb. Technol. 13: 158–163.

6 Poutamen, K. and M. Sundberg. 1988. An acetyl esterase of *Trichoderma reesei* and its role in the hydrolysis of acetyl xylans. Appl. Microbiol. Biotechnol. 28: 419–424.

7 Priest, F.G., C.T. Goodfellow and C.A. Todd. 1988. A numerical classification of the genus *Bacillus*. J. Gen. Microbiol. 134: 1847–1882.

8 Schär, H.P., D. Gyrax, G.M. Ramos Tombo and D. Ghisalba. 1988. Purification of an acetyl specific carboxylesterase from *Nocardia mediterranei*. Appl. Microbiol. Biotechnol. 27: 451–456.

9 Sneath, P.H.A. 1986. Endospore forming Gram positive rods and cocci. In: Bergey's Manual of Systematic Bacteriology (9th Edn.), Vol. II (Sneath P.H.A., ed.), pp. 1104–1139. The William and Wilkins Co., Baltimore.