Production of *Pseudomonas* Cells Having a High Fumarate Hydratase Activity

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L(-)Malic acid has found many uses in the food industries as a flavoring and acidulating agent (Irwin *et al.*¹⁾). Our laboratory is engaged in the study of enzymes insolubilized by entrapment into fibres (Dinelli²⁾). The production of L(-)malic acid by the enzymic hydration of fumaric acid, catalyzed by fibre-entrapped Fumarate Hydratase (FH) was investigated (Mosti *et al.*³⁾). In this communication we report on the production of microbial cells having a high FH activity for the preparation of the enzyme to be entrapped.

Screening for FH producing strains

Thirty five bacterial strains, which had been isolated from spoiled milk, were cultivated in shaker flasks containing medium M9F/Y.E. having the following composition (g/liter): Na₂HPO₄·2H₂O, 7.5; KH₂PO₄, 3.0; NH₄Cl, 1.0; NaCl 0.5; MgSO₄·7H₂O 0.2; fumaric acid (neutralized to pH 7.0 with NaOH) 10.0 and Yeast Extract, 1.0. The pH of the medium was adjusted to 7.0.

The FH activity of the cells was determined according to the method originally proposed by Racker,⁴⁾

based on the increase of the Optical Density at 240 nm, due to transformation of L(-) malic acid into fumaric acid. The cells were collected by centrifugation, washed with 0.1 M Na phosphate buffer pH 7.4 and disrupted by sonication at a temperature below 8°C. The cell-free extracts were kept at 4°C, since it was found that samples kept at -20°C underwent considerable inactivation. For the assay a 0.1 M solution of L(-) malic acid, neutralized to pH 7.4 with NaOH, was mixed with an equal amount of 0.1 M Na phosphate buffer pH 7.4. Three ml of this mixture were placed in both the sample and reference cuvettes (1 cm) of a recording double-beam spectrophotometer (Beckman DB-GT) and the reaction was started by the addition of 10 µl of the cell-free extract (when more active extracts were obtained later during this study, it was found that dilution with buffer caused inactivation of the enzyme, but that samples could be stabilized by diluting with buffer containing 2.5×10^{-3} M L(-) malic acid). The increase in optical density (O.D.) was recorded for 2 min: one Racker Unit (RU) of FH activity was taken as the amount of enzyme that caused, in 1 min at 25°C an increase of 0.001 Absorbance units: the ratio (RU/O.D.) of the activity (RU/ml) to the optical density of the culture was taken as an approximate measure of the specific activity of the cells.

The highest FH activity was found in strain SP 744, which was identified, on the basis of its morphological and cultural characteristics, as very similar to *Pseudomonas taetrolens*, from which it differs only in the metabolism of lactose. The strain was maintained by monthly transfers on Nutrient Agar.

Study of the culture medium

An exponentially growing shake flask culture of strain SP 744 on medium M9F/Y.E. was used as inoculum for some 500 ml shake flasks containing 100 ml of different media, as indicated in Table I. After

TABLE I. FH PRODUCTION BY STRAIN SP 744 IN DIFFERENT MEDIA

Culture medium		pH		O.D. (1:10)	Activity RU/ml	Sp. Act. RU/O.D.
		Initial	Final			
M9F/Y.E.		7.0	8.6	0.210	975	387
"	+ Trace elements ^{a)}	7.0	8.6	0.218	1,240	568
"	+ 20 g/l beet molasses	7.0	8.6	0.275	1,650	600
"	+ 10 g/l glucose	7.0	6.7	0.178	850	478
"	+ 10 g/l CSL ^{b)}	7.1	8.5	0.185	1,100	595
"	+ 5 g/l peptone	7.0	8.6	0.190	1,000	527
F8 ^{c)}		7.0	8.5	0.265	2,825	1,068

^{a)} Dworkin *et al.*⁵⁾

^{b)} Corn steep liquor was heat-treated as follows: the required quantity of CSL was diluted 1:3 with water, the pH was raised to 7.0 with 20% NaOH, the suspension was heated under stirring and kept at the boiling point for 10 min. After addition of 5% w/v of Celite, the hot suspension was filtered through a Buchner funnel and the residue washed with water. The filtrate was used.

^{c)} CSL, 50 g/l; fumaric acid, 10 g/l.

16 hr of incubation at 25°C on a rotary shaker, the cultures were examined for pH, growth (optical density as measured on 1:10 dilutions of the broth) and enzyme activity (expressed as RU/ml of culture). Since the most interesting results had been obtained in culture media containing considerable amounts of complex ingredients such as molasses and CSL, their use in culture media for FH production was further investigated: it was found that on medium F15, containing 50 g/liter CSL and 5 g/liter fumaric acid or on a medium containing 50 g/liter CSL and 20 g/liter molasses (no fumaric acid), activities of the order of 3.200 RU/ml could be obtained.

Further experiments showed that fumaric acid had no effect on growth and a slight, if any, effect on FH production: its presence seems however to favour the stability of the enzyme produced. The effect of different combinations of C and N sources (fumaric

acid, glucose, yeast extract, yeast autolysate, CSL, malt extract, casein hydrolysate, Proflo, Pharmamedia, peptone and Vegedor, a vegetable protein hydrolysate) was then examined: the highest production of FH was obtained when growing strain SP 774 on medium F16, containing (per liter) 50 g CSL, 10 g glucose and 5 g fumaric acid: in shake flasks FH activities of more than 6.000 RU/ml were obtained.

Effect of some physico-chemical conditions on FH production

The effect of the incubation temperature has been examined: as shown in Table II, at 30°C both growth and FH production are strongly inhibited. Experiments in stirred jar fermentors confirmed that the optimum incubation temperature for FH production is between 25 and 28°C.

Preliminary experiments in shake flasks, in which the

TABLE II. EFFECT OF THE INCUBATION TEMPERATURE OF FH PRODUCTION

Incubation temperature, °C		pH	O.D. (1:10)	RU/ml	RU/O.D.
Pre-culture ^{a)}	Production ^{b)}				
25°	25°	8.35	0.275	3,180	1,150
25°	30°	8.65	0.149	950	640
30°	25°	8.40	0.275	2,870	1,040
30°	30°	8.60	0.185	1,350	730

^{a)} Shake flasks with medium M9F/Y.E.

^{b)} Shake flasks with medium F15.

FIG. 1. New Brunswick Jar Fermentors of 7.5 l Capacity, Containing 4.5 l of Medium F 15, were Incubated at 28°C, with an Aeration Rate of 0.5 v/v/min and a Stirrer's Speed of 500 and 700 rpm. Under the conditions described, the OAR values were 0.25 and 1.70 mm O₂/l/min.

□ 500 rpm (OAR=0.25); ○ 700 rpm (OAR=1.70). —, pH and optically density at 550 nm, measured on 1:10 dilution of the culture samples; ···, enzyme activity (RU/ml).

aeration, as measured by the Oxygen Absorption Rate (OAR), was varied by using different liquid volumes in 500 ml Erlenmeyer flasks, had shown that at higher OAR values more FH activity (RU/ml) could be obtained, but that the cells had a lower specific activity (RU/O.D.). The OAR values (mm O₂/l/min) were determined by the sulfite oxidation method according to Corman *et al.*⁶⁾ The effect of the OAR in stirred jar fermentors is illustrated in Fig. 1. It was found in further experiments that it was unnecessary to use conditions which gave OAR values above 1.70 mm O₂/l/min. During cultivation of strain SP 744 both an synthetic and complex media containing fumarate, the pH of the culture rises to alkaline values. It was found that preventing the pH value of the growing culture from rising above 7.0, by means of automatic pH control, production of FH is about 10% higher as UR/ml and even higher as specific activity of the cells (UR/O.D.), since growth was slightly lower under controlled pH conditions.

REFERENCES

- 1) W. E. Irwin, L. B. Lockwood and M. F. Zienty in Kirk-Othmer, "Encyclopedia of Chemical Technology," 2nd edition, Vol. 12, 1967, p. 837~849.
 - 2) D. Dinelli, "Process Biochemistry," Aug. 1972, p. 9~12.
 - 3) R. Mosti, W. Marconi and F. Morisi, Communication presented at the FEBS Special Meeting Dublin, 15~19 April, 1973.
 - 4) E. Racker, *Biochim. Biophys. Acta*, **4**, 211 (1960).
 - 5) D. Dworkin and J. W. Foster, *J. Bact.*, **72**, 646 (1956).
 - 6) J. Corman, H. M. Tsuchiya, H. J. Koepsell, R. G. Benedict, S. E. Kelley, V. H. Feger, R. G. Dworschack and R. W. Jackson, *Appl. Microbiol.*, **5**, 313 (1957).
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