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Stimulation of *Erwinia* sp. Fumarase and Aspartase Synthesis by Changing Medium Components

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Abstract

The optimal concentrations of nutrient medium components, aeration conditions, and pH providing for maximum biomass yields, as well as fumarase and L-aspartase activities, during submerged cultivation of *Erwinia* sp. were determined. The data showed that different concentrations of carbon source (molasses) and pH of the nutrient medium were required to reach the maximum fumarase and L-aspartase activities. Calculations performed by application of the additive lattice model suggested that the combination of these optimized factors would result in 3.2-, 3.4-, and 3.8-fold increases as compared to the experimental means in *Erwinia* sp. biomass, and L-aspartase and fumarase activities, respectively. The conditions of the fumaric acid biotransformations into L-malic and L-aspartic acids were optimized on the basis of intact *Erwinia* sp. cells, a fumarase and L-aspartase producer. In the cases of fumarate transformation into L-malic acid and of fumarate transformation into L-aspartic acids, fumarase and L-aspartase activities increased 1.5- and 1.7-fold, respectively. The experimental data were consistent with these estimates to 80% accuracy. In comparison with the additive lattice model, the application of polynomial nonlinear model allowed the between-factor relations to be considered and analyzed, which resulted in 1.1-, 1.27-, and 1.1-fold increases in *Erwinia* sp. biomass and fumarase and L-aspartase activities for the case of cultivation. In the case of fumarate transformation

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into L-malic acid, this model demonstrated a 1.7-fold increase in fumarase activity, whereas during fumarate transformation into L-aspartic acid no significant change in aspartase activity was observed.

Index Entries: *Erwinia*; fumarase; aspartase; additive lattice model; polynomial nonlinear model.

Introduction

Fumarase (fumarate hydratase; EC 4.2.1.2) and L-aspartase (L-aspartate ammonia lyase; EC 4.3.1.1) enzymes realize fumarate biotransformation into L-malic and L-aspartic acids, respectively *(1–4)*. When cells are used as biocatalysts, the enzyme producers as well as the cultivation and transformation conditions are of particular importance. Often, the same strain simultaneously displays high activities of several enzymes. In this case, it is necessary to select cultivation conditions providing for a targeted synthesis of an enzyme with high activity.

Optimization of fermentation media components, as well as microbial growth and substrate transformation conditions, is required to make the microbiologic acid synthesis process efficient. However, this is usually based on numerous experiments. The application of mathematical methods of experimental design allows both the number of experiments to be decreased and adequate mathematical modeling of these processes on the basis of an analysis and mathematical processing of experimental data that makes possible the prediction of conditions of targeted change in parameters and criteria *(5–7)*.

The goal of the present work was to determine the optimal concentration of nutrient medium components, aeration conditions, and pH providing for maximum biomass, and fumarase and L-aspartase activities during submerged cultivation of *Erwinia* sp. We also aimed to determine the optimal conditions for fumarate transformation by intact *Erwinia* sp. cells, a producer of fumarase and L-aspartase, into L-malic and L-aspartic acids, respectively.

Materials and Methods

Inoculum and Growth Conditions

The strain *Erwinia* sp., with high fumarate hydratase and L-aspartate ammonia lyase activities, was used. A 12- to 14-h-old culture grown in 500-mL flasks containing 80 mL of a nutrient medium (5% hydrolyzed protein-vitamin concentrate, 1% molasses, both from industrial origin; and 1% sodium or ammonium fumarate and polyvinyl chloride (PVC), from Merck, Darmstadt, Germany), pH 7.0–7.2, at 28–30°C in an orbital shaker (Lab-line, Melrose Park, IL) at 250 rpm used as a 1% inoculum was introduced. Submerged bacteria cultures were grown in 500-mL Erlenmeyer flasks containing 30–80 mL of nutrient medium at 28–30°C over 12–14 h at 250 rpm. While optimizing the cultivation conditions, the component con-

centrations, pH, and medium volume (percent) varied in the ranges experimentally determined in preliminary experiments: 3 to 7% PVC, 0.5 to 1.7% molasses, 0.5 to 1.8% fumarate, pH 7.0–8.8, and 30–80 mL of the medium. The optical density of the culture was determined at 495 nm (LKB 4050 Biochrom spectrophotometer; Cambridge, UK) and then converted into dry weight (grams/liter) by means of a calibration curve at 540 nm *(8)*.

Enzyme Activities

Fumarase Activity

Biomass of 12- to 14-h-grown cultures was spun down at 6000*g* for 30 min. Cells (200 mg) were placed in a 30°C bath in 5 mL of 1 *M* sodium fumarate solution (pH 7.0) containing 0.001 *M* $MgCl_2$ in a shaker. Fumarase activity was determined by measuring the absorbance with a spectrometer while taking into account the molar extinction coefficient for fumarate (2500 $M^{-1}cm^{-1}$) at 240 nm *(9)*. The amount of L-malic acid was determined colorimetrically according to the method of Goodman and Stark *(10)*. Fumarase activity was expressed in millimoles per hour per gram of crude cells.

Aspartase Activity

Aspartase activity was determined under the same conditions by incubating 50 mg of cells in 5 mL of 1 *M* ammonium fumarate solution (pH 8.5). The reaction mixture was separated by thin-layer chromatography on Silufol plates with propanol:ammonia (7:3) as a solvent system. The plates were developed with 2% ninhydrin solution in acetone. Spots of L-aspartic acid were eluted with 60% ethanol supplemented with 0.1% $CdCl_2$ to measure the eluate's OD at 500 nm. The concentration of L-aspartic acid was determined from a calibration curve. Aspartase activity was expressed in millimoles per hour per gram of crude cells. The total enzymatic activity was calculated as the activity of crude cells (grams) produced in 1 L of fermentation medium and expressed in millimoles per hour.

Oxygen Measurements

The rate of oxygen uptake in the medium was measured using the sodium sulfite assay according to Yakovleva et al. *(11)*. Related to the volume of the medium, the rates were 50, 41, 35, 30, and 22 mmol/(L·h) (O_2) in 30, 40, 50, 60, and 80 mL of medium, respectively.

Experimental Design

The experiments were performed according to an orthogonal multilevel design to determine the optimal nutrient medium composition, aeration conditions, and pH. Five factors were varied at three levels in the case of the cultivation process, and four factors were varied at four levels in the case of the transformation process *(6)*. Approximating models of the cultivation-additive lattice model *(6)* and polynomial approximation with non-

Table 1
Optimization of Cultivation Conditions PVC, Molasses, Fumarate, pH, and Volume of Medium

	Cultivation condition								
	Variant I			Variant II			Variant III		
Factor	1	2	3	1	2	3	1	2	3
PVC (%)	3.0	4.5	6.0	4.5	5.0	5.5	6.0	6.5	7.0
Molasses (%)	0.5	1.0	1.5	1.5	2.0	2.5	1.2	1.7	2.2
Fumarate (%)	0.5	1.0	1.5	0.3	0.6	0.9	1.2	1.5	1.8
pH	7.2	8.5	9.8	6.8	7.5	8.2	7.3	8.0	8.7
Volume of medium (mL)[a]	40	60	80	30	40	50	30	40	50

[a]In 500-mL flasks in an orbital shaker at 250 rpm.

linear regression equation *(7)* were obtained from mathematical processing of experimental data.

Results and Discussion

The aim of the present work was to determine the optimal cell growth and enzyme production conditions for a strain of *Erwinia* sp. by changing the nutrient medium content. A strain of *Erwinia* sp. was selected from a number of microorganisms of the gena *Escherichia*, *Erwinia*, *Alcaligenes*, *Pseudomonas*, and *Citrobacter* for the synthesis of fumarase and L-aspartase (data not shown).

Within the ranges of input factors determined previously, a simultaneous experiment was performed according to an orthogonal multilevel design: at three levels of 18 individual experiments each ($18 \times 3 = 54$ experiments total) in three variants varying five factors in the case of the cultivation process; and at four levels of 16 individual experiments each ($16 \times 4 = 64$ experiments total) in two variants varying four factors in the case of the transformation process. The values of the input factors are given in Tables 1 and 2. Experimental data were processed separately: in the case of the cultivation process with respect to the biomass cell yield, and fumarase and L-aspartase activities; in the case of fumarate transformation into L-malic acid with respect to the fumarase activity yield and in the case of fumarate transformation into L-aspartic acid with respect to the L-aspartase activity yield.

Additive Lattice Model

An estimate of Y_i for each combination was calculated according to ref. *6*:

$$\hat{Y} = b_0 + f_1[X_1] + f_2[X_2] + \ldots + f_n[X_n] \quad (1)$$

in which n is the number of factors.

Since the approximating model is additive, the overall optimum of the process as a whole (Y) is determined as the sum of local optimums for each

Table 2
Study of Substrate Concentration, Biomass Concentration, pH, and Temperature in Fumarate Transformation into L-Malic Acid and L-Aspartic Acid

	Conditions of fumarate transformation into L-malic acid			
Factor	1	2	3	4
Substrate concentration, *s* (M)	0.6	0.8	1.0	1.2
Biomass concentration (mg/mL)	50	100	150	200
pH	6.5	7.0	7.5	8.0
Temperature (°C)	25	30	35	40
	Conditions of fumarate transformation into L-aspartic acid			
Factor	1	2	3	4
Substrate concentration, *s* (M)	0.6	0.8	1.0	1.2
Biomass concentration (mg/mL)	40	60	80	100
pH	7.5	8.0	8.5	9.0
Temperature (°C)	30	35	30	45

factor *(1)*. The value of coefficients of the additive lattice model is calculated as follows:

$$b_0 = \left(\sum_{j=1}^{N} Y_j\right)/N, \quad b_{ik} = \left(\sum_{j=1}^{N} Y_{ij}^{(k)}\right)/(N/m) - b_0 \tag{2}$$

in which N is the total number of the experiments in the design, m is the number of the levels of factors, and $\sum_{j=1}^{N} Y_{ij}^{(k)}$ is the sum of output in those variants of design in which the i-factor is on the k level.

The confidence intervals (CIs) of the coefficient estimates are determined as follows:

$$E = t\sigma_Y\sqrt{m/N} \tag{3}$$

in which Student's t-test was determined for the number of degrees of freedom used to calculate the reproducibility dispersion of the output index σ_Y^2, which was estimated as follows:

$$\sigma_Y^2 = \frac{\sum_{j=1}^{N} (Y_j - \hat{Y}_j)^2}{N - p - 1} \tag{4}$$

in which $\hat{Y}_j$ is the calculated yield of output on the j variant, $N - p - 1$ is the number of degrees of freedom.

The calculated optimal levels of individual factors and their effects on the cultivation and transformation processes are given in Tables 3 and 4.

Table 3
Calculated Optimal Values of Cultivation Conditions

	Cultivation condition					
			Total activity (mmol/h)			
	Biomass (g/mL) (0.919)[a]		Fumarase (6.42)[a]		Aspartase (9.08)[a]	
Factor	Calculated	Effect	Calculated	Effect	Calculated	Effect
PVC (%)	6.0	0.42	6.0	3.32	6.0	5.03
Molasses (%)	1.5	0.35	1.2	2.71	1.5	3.15
Fumarate (%)	1.5	0.71	1.5	4.53	1.5	6.08
pH	7.3	0.10	7.3	3.91	8.0	2.26
Volume of medium (mL)[b]	40	0.48	40	3.52	40	5.21

[a]Mean experimental values.
[b]In 500-mL flasks in an orbital shaker at 250 rpm.

Table 4
Calculated Optimal Values of Fumarate Transformation into L-Malic Acid and L-Aspartic Acid

	Conditions of fumarate transformation into L-malic acid	
	Fumarase activity (mmol/[h·g]) (8.76)[a]	
Factor	Calculated	Effect
Substrate concentration, *S* (*M*)	0.6	0.994
Biomass concentration (mg/mL)	100	2.5
pH	6.5	0.952
Temperature (°C)	40	1.8
	Conditions of fumarate transformation into L-aspartic acid	
	L-Aspartase activity (mmol/[h·g]) (11.68)[a]	
Factor	Calculated	Effect
Substrate concentration, *S* (*M*)	0.8	0.51
Biomass concentration (mg/mL)	60	1.54
pH	9.0	2.71
Temperature (°C)	35	0.882

[a]Mean experimental values.

The average yield of *Erwinia* sp. in the cultivation process increased 3.2-fold compared with the experimental value; the activities of L-aspartase and fumarase increased 3.4- and 3.8-fold, respectively. As shown in Table 2,

the maximal values of L-aspartase and fumarase activities required different concentrations of molasses and pH. Fumarase activity reached its maximum at 1.2% molasses and pH 7.3, and L-aspartase activity at 1.5% molasses and pH 8.0. The specific activities of L-aspartase and fumarase increased 3.39- and 3.79-fold, respectively.

In the case of the fumarase transformation, fumarate hydratase activity increased 1.5-fold compared with the mean experimental value, whereas in the case of L-aspartase transformation, ammonia lyase activity increased 1.7-fold compared with the mean experimental value. The experimental data were consistent with the estimates mentioned above at 80% accuracy. Thus, for *Erwinia* sp., biomass, and aspartase and fumarase activity, compared with the experimental means were $K \approx 2.56$, 2.72, and 3.04, respectively, and the specific activities of L-aspartase and fumarase were $K \approx 2.71$ and 3.03, respectively, whereas in the case of the transformation process the increases in fumarase and L-aspartase activities were $K \approx 1.2$ and 1.4, respectively.

Polynomial Approximation

The additive polynomial model used has the following general form *(7)*:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ijk} X_i X_j X_k + \sum \beta_{ii} X_i^2 + \sum \beta_{iii} X_i^3 + e \qquad (5)$$

in which Y is the dependent variable or the estimated yield, $(X_1, X_2 \ldots X_p)$ are independent variables or input factors, $(\beta_0, \beta_i, \beta_{ij}, \beta_{ijk}, \beta_{ii}, \beta_{iii})$ are unknown coefficients, and e is the error of approximation.

As far as the degree of correlation Y for pair combinations of the input factors (r_{ij}), their triple combinations (r_{ijk}), squares (r_{ii}), and cubes (r_{iii}) depend on the transfer of $r_{Yij} \neq r_{Yji}$; $r_{Yijk} \neq r_{Yikj} \neq r_{Yjik} \neq r_{Yjki} \neq r_{Ykij} \neq r_{Ykji}$. Therefore, by canceling the number of the unknown coefficients according to the criterion $\max r_{Yij}$, $\max r_{Yijk}$ and providing for $(P_1 + 1)$ bonds between the quantity m of the unknown coefficients and P_1 introduced again into the input variables Z, one obtains the model of the multiple linear regression in the following from:

$$Y = \beta_0 + \beta_1 Z_1 + \beta_2 Z_2 + \ldots + \beta_{P1} Z_{P1} + e \qquad (6)$$

The vector $\beta = (\beta_0, \beta_1, \beta_2, \ldots \beta_{P1})'$ is determined by solving the system of normal equations $(Z'Z)^{-1}\beta = Z'Y$, which has the form

$$\hat{\beta} = (Z'Z)^{-1}Z'Y \qquad (7)$$

in which Z' is the scheme matrix with a dimension of $n \times (P_1 + 1)$.

Both the values of p and the degree of correlation of the yield Y for pair combinations of the input factors (r_{ij}), their triple combinations (r_{ijk}), and squares (r_{ii}) are defined as a result of correlation analysis *(7,12,13)*.

The values of individual correlation coefficients were calculated according to

$$\overline{Z}_i = \frac{1}{n}\sum_{k=1}^{n} Z_{ik}, \quad \overline{Y} = \frac{1}{n}\sum_{k=1}^{n} Y_k$$

$$S_i^2 = \frac{1}{n-1}\sum_{k=1}^{n} (Z_{ik} - \overline{Z}_i)^2$$

$$S_{ij} = \frac{1}{n-1}\sum_{k=1}^{n} (Z_{ik} - \overline{Z}_i)(Z_{jk} - \overline{Z}_j) \tag{8}$$

$$r_{ij} = \frac{S_{ij}}{S_i \cdot S_j}$$

$$S_{yi} = \frac{1}{n-1}\sum_{k=1}^{n} (Y_k - \overline{Y})(Z_{ik} - \overline{Z}_i)$$

in which $i = 1, 2, \ldots, P_1$, and n is the number of experiments.

For pair and triple combinations the recurrent ratios were used:

$$r_{Yij} = \frac{r_{Yi} - r_{Yj} \cdot r_{ji}}{\sqrt{1 - r_{Yi}^2} \cdot \sqrt{1 - r_{ji}^2}}$$

$$r_{Yijk} = \frac{r_{Yij} - r_{Ykj} \cdot r_{ikj}}{\sqrt{1 - r_{Ykj}^2} \cdot \sqrt{1 - r_{ikj}^2}} \tag{9}$$

The dispersion analysis for the model of multiple linear regression was carried out according to the following basic parameters:
The estimate of dispersion of errors or the residual mean square of error

$$MS_{resid.} = \frac{SS_{resid.}}{n - P_1 - 1} = \frac{\sum_{i=1}^{n} (Y_i - \widehat{Y}_i)^2}{n - P_1 - 1} \tag{10}$$

The mean square dependent on the regression

$$MS_D = \frac{SS_D}{P_1} = \frac{\sum_{i=1}^{n} (\widehat{Y}_i - \overline{Y})^2}{P_1} \tag{11}$$

The square of multiple correlation coefficient or the determination coefficient

$$R^2 = \frac{SS_D}{SS_{corr.}} = \frac{\sum_{i=1}^{n} (\widehat{Y}_i - \overline{Y})^2}{\sum_{i=1}^{n} (Y_i - \overline{Y})^2} \tag{12}$$

The estimates of standards deflexion $\hat{\beta}_j$ for β_j were obtained by means of CIs

$$\hat{\beta}_j \pm Se(\hat{\beta}_j) \cdot t_{1-\alpha/2}\,(n - P_1 - 1) \tag{13}$$

in which

$$Se(\hat{\beta}_j) = \sqrt{\frac{SS_{resid.}/(n - P_1 - 1)}{\sum_{i=1}^{n} (Z_{ji} - \overline{Z}_j)^2}}$$

and the testing of hypothesis concerning the coefficients β_j correlative with $F = (MS_D/MS_{resid.})$.

The coefficients of yield estimates were obtained for the following input factors: X_1, the content of hydrolyzed PVC (%); X_2, molasses concentration (%); X_3, Na or NH_4 concentration (%); X_4, pH of the nutrient medium; and X_5, volume of the medium (mL) or the corresponding oxygen uptake rate (mmol/[L·h]) in 500-mL Erlenmeyer flasks in a shaker at 250 rpm.

The null hypothesis H_0 ($\beta_i = 0$; $\beta_{ij} = 0$; $\beta_{ijk} = 0$; $\beta_{ii} = 0$; $\beta_{iii} = 0$, in which $i = 1, 2, \ldots, 5$; $j = 2, \ldots, 5$; $k = 3, 4, 5$) was rejected in the cases of joint yields, since $F_{(99.9/100)}(5.48) = 4.91$ *(7)*. A comparison of this value with $F(5.48)$ for individual yields demonstrated that $F_{(99.9/100)}(5.48) = 45.45 > 4.91$ for biomass yield, $F_{(99.9/100)}(5.48) = 27.7 > 4.91$ for fumarase specific activity, and $F_{(99.9/100)}(5.48) = 8.72 > 4.91$ for L-aspartase specific activity.

The standard error of the estimation was 0.11, 1.45, and 1.85, respectively, for three yields, and the determination coefficient R^2 indicating the degree of the quality of fit or degree of approximation of the nonlinear model was 0.88, 0.73, and 0.65, respectively. The quantity R^2 is the square of the multiple coefficient of correlation $r_{y,x_1,x_2,x_3,x_4,x_5} = R$; therefore, for three yields $R \approx 0.94$, 0.85, and 0.81, respectively.

The nonlinear polynomial model predicted the following maxima, which are higher than the experimental means: $K \approx 2.83$ for biomass yield, $K \approx 3.85$ for fumarase specific activity, and $K \approx 2.99$ for L-aspartase specific activity. Comparison of the presented values with the corresponding ones derived from the additive lattice model showed that the application of the nonlinear polynomial model resulted in a 1.1-, 1.27-, and 1.1-fold increase, respectively, for all yields.

The dependencies of maximum values of the data from each input factor for any possible combination of other factors are shown in Figs. 1 and 2.

Analysis of the correlation of the cultivation process led to the following main conclusions for three experimental factors (biomass, and fumarase and L-aspartase specific activities). A relatively high correlation effect was demonstrated.

1. Biomass yield.
   a. Oxygen uptake rate (OUR) (0.72).
   b. OUR squared (0.59).
   c. Na or NH_4 fumarate, pH of the medium, and OUR cubed (0.67, 0.58, 0.95, respectively).
   d. OUR with fixing: content of hydrolyzed PVC (0.85), molasses concentration (0.72), fumarate concentration (0.8), and pH of the medium (0.7).

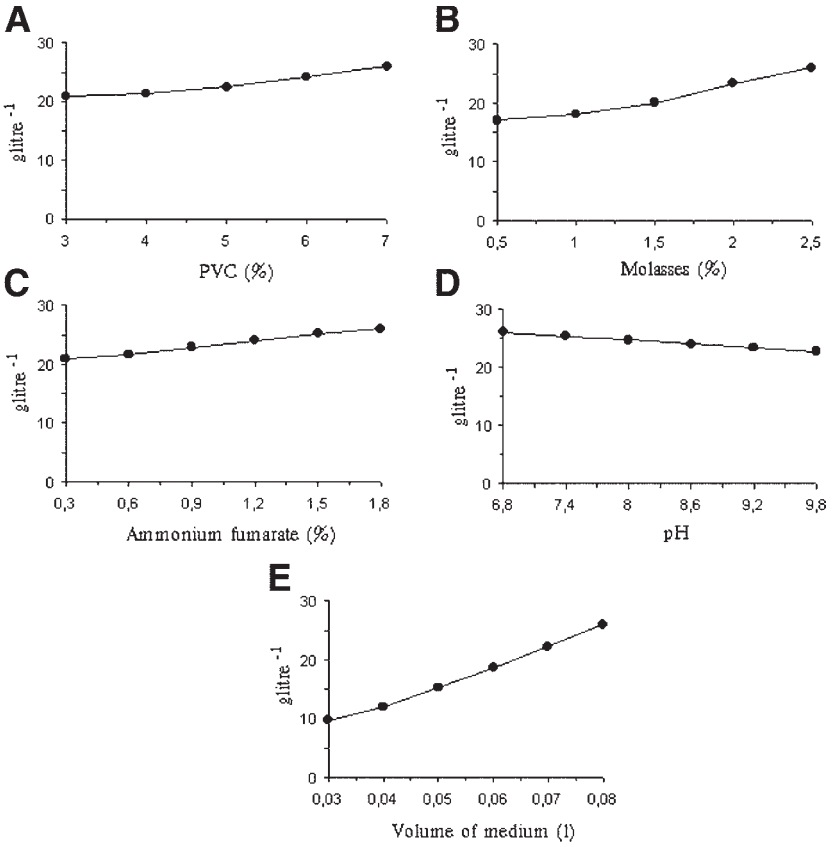

Fig. 1. Influence of changing **(A)** PVC, **(B)** molasses, **(C)** ammonium fumarate, **(D)** pH, and **(E)** volume of medium on biomass. All the factors showed a positive effect on biomass except a change in pH.

e. OUR with pair fixing: content of hydrolyzed PVC and molasses concentration (0.84), content of hydrolyzed PVC and Na or NH_4 fumarate (0.87), content of hydrolyzed PVC and pH of the medium (0.84), Na or NH_4 fumarate and molasses concentration (0.84), Na or NH_4 fumarate and pH of the medium (0.8).

2. Fumarase specific activity.
   a. Content of hydrolyzed PVC (0.62).
   b. Molasses concentration and Na or NH_4 fumarate cubed (0.65 and 0.68, respectively).
   c. Content of hydrolyzed PVC under fixing of pH of the medium (0.6).
   d. Content of hydrolyzed PVC under pair fixing molasses concentration and pH of the medium (0.6).
3. L-Aspartase specific activity.
   a. Na or NH_4 fumarate cubed (0.62).

Comparison of the experimental and calculated data demonstrated that the nonlinear polynomial model gives on average about

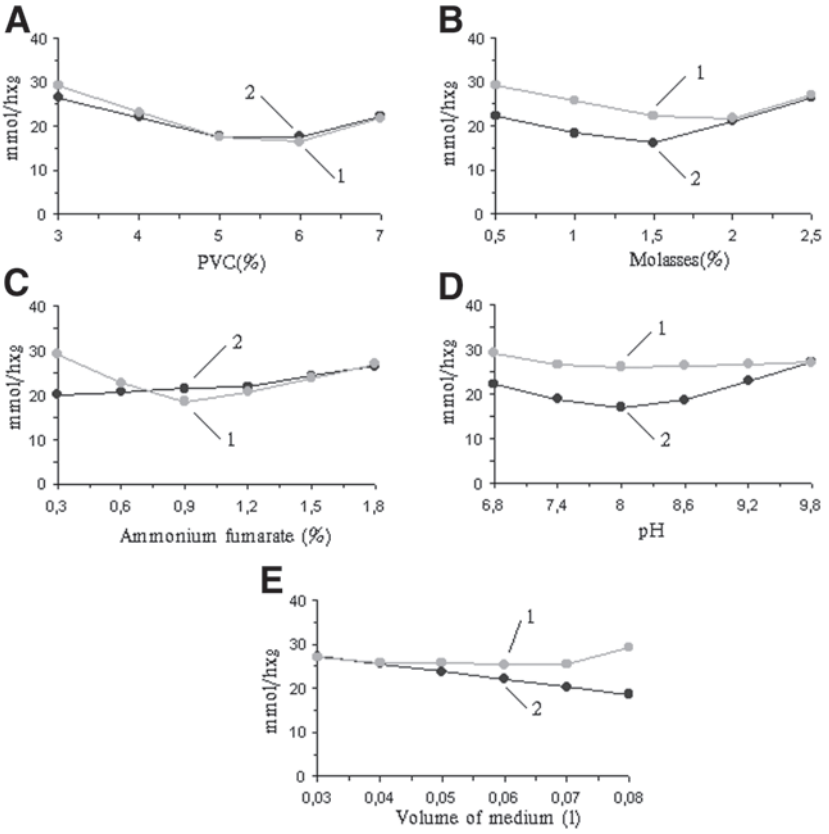

Fig. 2. Influence of changing **(A)** PVC, **(B)** molasses, **(C)** ammonium fumarate, **(D)** pH, and **(E)** volume of medium on L-aspartase (1) and fumarase (2). All the factors showed similar effects on both activities.

82 and 81% approximation of the fumarate hydratase L-aspartate ammonia lyase activities. The null hypothesis H_0 ($\beta_i = 0$; $\beta_{ij} = 0$; $\beta_{ijk} = 0$; $\beta_{ii} = 0$; $\beta_{iii} = 0$, in which $i = 1, 2, 3, 4$; $j = 2, 3, 4$; $k = 3, 4$) was rejected in the cases of joint yields, since $F_{(99.9/100)}(4.11) = 10.35$ *(7)*. A comparison of this value with $F(4.11)$ for individual yields demonstrated that $F_{(0.999)}(4.11) = 14.7 > 10.35$ for fumarate hydratase activity and $F_{(0.999)}(4.11) = 12.4 > 10.35$ for L-aspartate ammonia lyase activity. The standard error of the estimation was 1.5 for both fumarate hydratase activity and L-aspartate ammonia lyase activity, and the determination coefficient R^2 was 0.83 and 0.81, respectively. The multiple coefficient of correlation accounted for $R \approx 0.91$ in the case of fumarate hydratase activity, and $R \approx 0.90$ in the case of L-aspartate ammonia lyase activity. The nonlinear polynomial model predicted the following maximum increases compared to the experimental means: $K \approx 2.01$ for fumarate hydratase activity and $K \approx 1.22$ for L-aspartate ammonia lyase activity. Comparison of the presented values with those derived from the additive lattice model

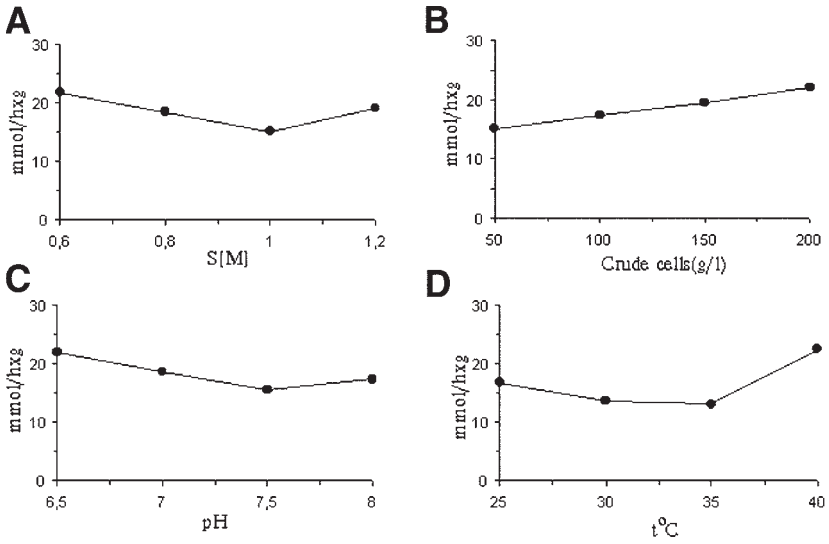

Fig. 3. Influence of **(A)** fumarate concentration, **(B)** biomass, **(C)** pH, and **(D)** temperature on fumarase activity.

showed that the application of the nonlinear polynomial model resulted in a 1.7-fold increase in fumarate hydratase activity, whereas this model did not demonstrate relevant improvements in the activity of L-aspartate ammonia lyase.

The dependencies of maximum values of both activities from the individual input factors for any possible combination of other factors are shown in Figs. 3 and 4.

Analysis of the transformation correlation suggests the following:

1. Correlation of fumarate hydratase activity was positive with respect to biomass concentration and temperature.
2. Correlation of L-aspartase ammonia lyase activity was also positive with respect to substrate concentration, pH of the medium, and temperature.
3. A positive correlation of fumarate hydratase activity with respect to substrate concentration was not demonstrated, and correlation of L-aspartase ammonia lyase activity with respect to substrate concentration was negligible.
4. Correlation of fumarate hydratase activity with respect to biomass concentration reached its peak (0.56) at constant temperature and substrate concentration; a positive correlation of L-aspartase ammonia lyase activity with respect to biomass concentration was not demonstrated.
5. Correlation of fumarate hydratase activity was negative with respect to pH of the medium. By contrast, correlation of L-aspartase ammonia lyase activity with respect to pH of the medium reached its peak

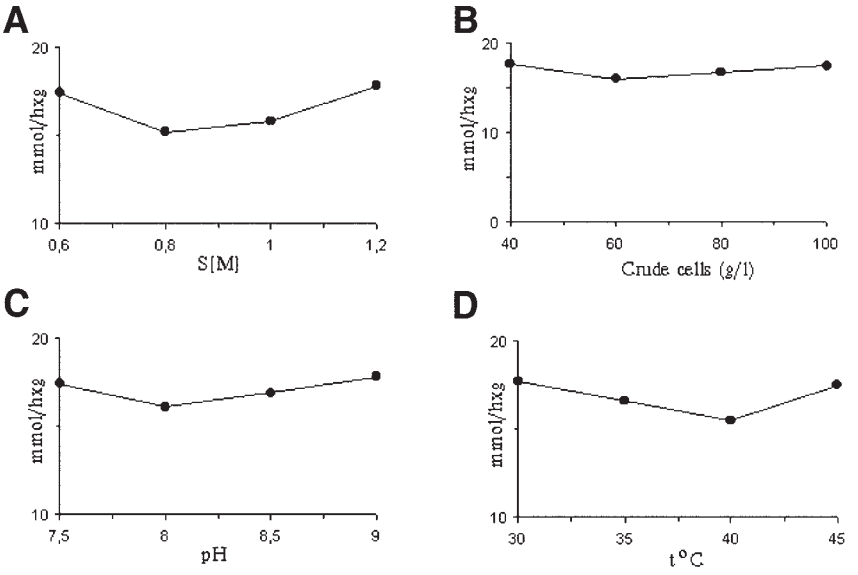

Fig. 4. Influence of **(A)** fumarate concentration, **(B)** biomass, **(C)** pH, and **(D)** temperature on L-aspartase activity.

(0.63) when substrate and biomass concentrations as well as temperature were fixed.

6. Correlation of fumarate hydratase activity with temperature reached its peak (0.53) when substrate concentration, biomass, and pH of the medium were constant, whereas correlation of the L-aspartase ammonia lyase activity did not demonstrate yield improvements under constant substrate concentration and pH of the medium.

Conclusion

Fumarate biotransformations into L-malic and L-aspartic acids were studied on the basis of intact *Erwinia* sp. cells, a producer of fumarase and L-aspartase. Fumarate transformation by *Erwinia* sp. proceeded without synthesis of any other coacids.

In comparison with the additive lattice model, application of the polynomial nonlinear model allowed between-factor relations to be considered and analyzed, which resulted in 1.7- and 1-fold increases in fumarase and L-aspartase activities, respectively, for the case of transformation, and 1.1-, 1.27-, and 1.1-fold increases in *Erwinia* sp. biomass, L-aspartase activity, and fumarase activity, respectively, for the case of cultivation.

Optimization of the concentration of the nutrient medium's components and cultivation conditions allowed the growth of cells to be intensified and aspartase and fumarase activities of the strain *Erwinia* sp. to be increased 3.2–3.8-fold during cultivation. This increase of nutrient consumption can be regulated and may be directed to the production of fumarase and L-aspartase by *Erwinia* sp.

References

1. Takato, I., Yamamato, K., Tosa, T., and Chibata, I. (1979), *Appl. Microbiol. Biotechnol.* **7,** 161–168.
2. Ertan, H. (1992), *Arch. Microbiol.* **158,** 42–47.
3. Flint, D. H., Emptage, M. H., and Guesh, J. R. (1992), *Biochemistry* **31,** 10,331–10,337.
4. Tosa, T., Sato, T., Nishida, Y., and Chibata, I. (1977), *Biochem. Biophys. Acta* **483,** 193–202.
5. Esgalhado, M. E., Caldeira, A. T., Roseiro, J. C., and Emery, A. N. (2002), *Biochem. Eng. J.* **12,** 181–192.
6. Biryukov, V. V. (1985), in *Optimization of Periodical Processes in Microbiological Synthesis*, Nauka, Moscow, pp. 18–52.
7. Afifi, A. and Azen, S. (1979), in *Statistical Analysis*, Academic, New York, pp. 141–194.
8. Meynell, G. and Meynell, E. (1965), *Theory and Practice in Experimental Bacteriology*, Cambridge, translated under the title "Eksperimental'naya microbiologiya," Mir, Moscow, 1967, p. 14.
9. Bagdasaryan, Z. N., Gukasyan, G. S., Abrahamyan, A. A., Aleksanyan, G. A., and Bagdasaryan, S. N. (1999), in *4th International Symposium on Biocatalysis and Biotransformations*, p. 30.
10. Goodman, A. E. and Stark, I. B. (1957), *Anal. Chem.* **29(2),** 283–287.
11. Yakovleva, V. I., Malofeev, I. V., Zueva, N. N., and Andreeva, A. P. (1979), *Prikl. Biokhim. Mikrobiol.* **15(3),** 328–336.
12. Smith, G. G. and Johnson, M. J. (1954), *Bacteriology* **68,** 346–352.
13. Deming, S. N. and Morgan, S. L. (1987), *Experimental Design: A Chemometric Approach*, Elsevier Science Publishers BV, Amsterdam.