Effect of Growth Temperature on Hydrolytic and Esterifying Activities from *Pseudomonas fragi* CRDA 037 Grown on Whey

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The production of hydrolytic and esterifying activities of *Pseudomonas fragi* CRDA 037 grown on a whey-based medium was investigated at different temperatures over time. The optimal temperature was found to be critical and different for the production of both activities. The highest hydrolytic activity was detected with bacteria cultivated at between 24°C (149.2 U/liter) and 27°C (133.8 U/liter), while the highest production of ethyl valerate (esterifying activity) was observed by using biomass grown at 15°C (0.75 U/liter). When the fermentation temperature was increased, the incubation time necessary to reach the maximal concentration of both activities was reduced. Studies of the thermostability of both activities showed that the hydrolytic activity was more stable than the esterifying activity at 15 and 24°C. Statistical analysis allowed the determination of the equations that predicted the production of hydrolytic and esterifying activities as a function of time and growth temperature. The optimal assay temperatures for the hydrolytic and esterifying activities were 37°C and 12 to 15°C, respectively.

Fruity aromas, due to fatty acid ethyl esters such as ethyl butyrate and ethyl hexanoate, have been detected in dairy products stored at low temperatures and spoiled by *Pseudomonas fragi* (3, 17). Although it may be deleterious to the food industry, *P. fragi* represents an obvious biotechnological interest. Indeed, this psychrotrophic microorganism hydrolyzes triglycerides of milk and butter in the 1,3-position and uses the low-molecular-weight fatty acids released to produce aroma compounds (11, 13, 21). Nevertheless, the enzymatic system involved in these reactions has been only partially identified. Endo- and exolipases have been purified (15, 16, 21) and may be responsible for ethyl ester formation. Intracellular esterases from *P. fragi* were also found to be involved in the biodegradation of esters (8, 19).

The aim of this work was to optimize the production of the hydrolytic and esterifying activities of *P. fragi* during the fermentation of whey. It has been reported that the optimal temperature for enzyme production by *Pseudomonas* sp. was lower than the optimal growth temperature (1, 18) and that lipase production did not increase concomitantly with the biomass. For instance, several studies with *Pseudomonas fluorescens* MF0 have shown that the optimal production of lipase, protease, and three periplasmic phosphatases occurred at 17.5°C, whereas its optimal growth temperature was ca. 30°C (4, 12). However, the culture temperature did not control the constitutive cell-bound esterase and cytochrome oxidase activities of *P. fluorescens* (12). These results indicate that the enzymatic regulation by temperature is complex and may take place at various levels. This hypothesis was challenged by Burini et al. (2), who cloned the lipase and acidic phosphatase genes in order to observe the effect of temperature. These authors demonstrated that the growth temperature regulation of those activities was posttranscriptional. Guillou et al. (5) also confirmed this conclusion and suggested that *P. fluorescens*

MF0 lipase was inductive and sensitive to catabolic repression and that growth temperature could modulate the lipase production.

The quantity of cellular proteins from *P. fragi* was found to increase when the fermentation temperature was lower than the optimal growth temperature (6). About 70 proteins that represent 13.5% of the total cellular proteins were screened. These proteins were classified into five classes according to the temperature of their production. According to their class, the rates of synthesis of the proteins were found to increase or decrease with temperature. The third class contained proteins with highest expression at temperatures ranging from 15 to 20°C. According to Mérieau et al. (12), Hébraud et al. (6), and Raymond et al. (18), the ester formation from *Pseudomonas* sp. was highest at 12°C, but its optimum growth temperature was close to 30°C. On the basis of on these findings, we investigated the production of hydrolytic and esterifying activities from *P. fragi* grown at between 12 and 30°C in order to demonstrate the importance of temperature on the production of these enzymes.

MATERIALS AND METHODS

Microorganism. Stock cultures of *P. fragi* CRDA 037 were prepared as described by Morin et al. (14).

Standardization of the inocula. A subculture was first prepared on brain heart infusion (BHI) agar and incubated at 30°C. After 24 h, the cells were suspended in 5 ml of sterile NaCl solution (0.85%) with an absorbance (A_{600}) adjusted to 0.2 ± 0.01 (Beckman Spectrophotometer DU-7; Beckman Instruments, Inc., Irvine, Calif.). Next, a 50-ml aliquot of BHI broth in a 250-ml Erlenmeyer flask was inoculated with the standardized bacterial suspension (1% [vol/vol]) and incubated at 30°C at 150 rpm for 24 h. Finally, an appropriate dilution of the 50-ml culture was performed in freshly prepared BHI broth in order to have an A_{600} of 0.2 ± 0.01 ; this was then used as an inoculum for the main culture.

Fermentation medium and culture conditions. The medium used for the production of *P. fragi* cells was composed of rehydrated whey (6% [wt/wt]) (Les Fromageries Saputo Ltée, St.-Hyacinthe, Quebec, Canada). The pH was adjusted to 8.0 with 6 N KOH prior to autoclaving at 118°C for 20 min. The culture medium was then supplemented with 0.1% butyric acid (vol/vol) and 0.2% food-grade ethanol 95% (vol/vol), and the pH was adjusted to 6.5 prior to inoculation of the culture with 1% standardized suspension (vol/vol). Cultures of 200 and 400 ml on whey in 1-liter Erlenmeyer flasks stirred at 150 rpm were incubated at each selected temperature.

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Harvesting of biocatalyst. The *P. fragi* resting cells used for the determination of the esterifying activity were harvested by centrifugation at 4°C at $17,700 \times g$ for 10 min (Beckman model J2-21; Beckman Instruments, Inc., Montreal, Quebec, Canada). They were then washed three times with potassium phosphate buffer (0.05 M, pH 7.5) and centrifuged as described above. Finally, 25% (wt/vol) bacterial suspensions in potassium phosphate buffer (0.05 M, pH 7.5) were prepared. For the determination of the hydrolytic activity, cells were harvested by centrifugation at $13,490 \times g$ for 5 min (Sigma Laboratory Centrifuge, model 113) and washed three times with sodium phosphate buffer (0.1 M, pH 7.0) before a 2.5% (wt/vol) cell suspension was prepared in the same buffer.

Esterifying activity. The esterifying activity was assayed in 250-ml Dreschel gas washing bottles outfitted with a sintered-glass gas inlet (porosity no. 3) and a gas outlet at 12°C for 6 h as described by Lamer et al. (9). The reaction mixture consisted of 90 ml of Tris-HCl buffer (0.1 M, pH 9.0), 10 ml of 25% cell suspension, 0.01 M valeric acid (Laboratoire Mat, Beauport, Quebec, Canada), and 0.02 M food-grade ethanol. The aeration of this system was set to 100 liters/min. Volatiles in the gas effluent were sampled for 2 min by connecting a stainless-steel tube (0.6-cm diameter by 17.8-cm length) filled with Tenax TA (60/80 mesh) to the reactor vent. For every assay of esterifying activity, three traps were collected and analyzed. The aroma compounds were desorbed from the Tenax traps by using a thermal AERO Trap Desorber 6000 (Tekmar, Cincinnati, Ohio) and injected into a Perkin-Elmer model 8320 gas chromatograph operating with a flame ionization detector under the conditions reported by Lamer et al. (9). The gas chromatograph analysis gave the amount of ethyl valerate in micrograms produced per 2 min. Based on an ethyl valerate calibration curve on traps (area versus ester concentration in micrograms), the esterifying activity of *P. fragi* was converted into micromoles of ethyl valerate formed per minute (one enzymatic unit) per liter of reaction volume with 25 g of bacterial cells per liter.

Hydrolytic activity. The hydrolytic-activity test was carried out in 96-well microplates. The hydrolytic activity of resting *P. fragi* cells was investigated with *p*-nitrophenyl valerate (Sigma Chemical Co., St. Louis, Mo.). The progress of *p*-nitrophenol release was measured at 405 nm (Lambda Reader EL 309 PE; Perkin-Elmer, Norwalk, Conn.). Stock solutions (48 mM) of *p*-nitrophenyl valerate were prepared in absolute ethanol and kept at 4°C for 1 week at most. They were diluted (1:100 [vol/vol]) just before the test in sodium phosphate buffer (0.1 M, pH 7.0). The reaction mixture consisted of 180 μ l of the substrate (0.48 mM) and 20 μ l of a 2.5% (wt/vol) cell suspension (22). A control reaction without the enzyme was performed in parallel to compensate for the spontaneous substrate hydrolysis. The temperature was set at 37°C and the agitation at 150 rpm. After 10 and 40 min of incubation, the A_{405} was measured as follows:

$$\Delta A_{405}/\text{minute} = \frac{(s_{40} - b_{40}) - (s_{10} - b_{10})}{30 \text{ min}}$$

where s is the A_{405} of the sample after 10 and 40 min, as indicated, and b is the A_{405} of the control reaction after 10 and 40 min, as indicated.

Using a standard curve of *p*-nitrophenol, the absorbance data were converted into the amount of enzyme that catalyzes the release of 1 μ mol of *p*-nitrophenol per min, i.e., into 1 enzymatic unit (U). In order to express the hydrolytic activity in enzymatic units per liter of reaction volume with 25 g of cells per liter the results (in enzymatic units) were multiplied by a factor 50,000. For every hydrolytic assay, seven replicates were performed.

Statistical analysis. The aim of the experiment was to determine which levels of the studied factors maximized the two enzymatic activities. The studied factors were the growth temperature and the fermentation time at which samples were obtained. For the hydrolytic activity, seven equally spaced levels of temperature ranging from 12 to 30°C were tested, and seven replications of the design were completed. For the esterifying activity, five equally spaced levels of temperature ranging from 12 to 24°C were tested, and three replicates, independent of the preceding seven ones, were performed.

Analysis of variance. Before the level(s) of temperature and time favoring high activities were determined, it was necessary to determine whether or not these factors and their interaction had an effect on the responses. The SAS system procedure MIXED was used to address this problem. PROC MIXED allows the analysis of repeated measurements and takes into account all of the observations even if there are missing values, whereas PROC GLM removes observations containing missing values. The temperature-sampling-time structure of the data varied with temperature, thus MIXED was the most appropriate procedure to use (10, 20).

Effects of temperature and time on the hydrolytic and esterifying activities. For each of the two activities, the second step of the statistical analysis was to build a second-order polynomial regression model describing the effect of the two independent variables on the response variable. Such a model is useful for prediction purposes or to determine the effects of the factors on the response when an analysis-of-variance model cannot be used, as for esterifying activity. The second-order polynomial regression can be written as follows:

$$\hat{Y} = b_0 + b_1T + b_{11}T^2 + b_2t + b_{22}t^2 + b_{12}Tt + \varepsilon$$

where $\hat{Y}$ is the predicted activity from the model; T and t represent the temperature and the time variables, respectively; and b_i , b_{ij} , and b_{ij} are the regression

coefficients estimated by the least-squares method. The assumptions underlying the analysis are that the errors are normally and independently distributed with a mean zero value and constant variance. In order to determine which model best fit the data, the PRESS (prediction sum of squares) statistic was computed. Each observation of the data set was successively removed from the model, and the model was fitted to the data. Then, a predicted value was obtained without this observation. This allowed the computation of the PRESS statistic. A PRESS statistic can be computed for each of the tested models. It permits the quantification of the difference between the observed and predicted values and therefore it permits the comparison of the fitted models. The PRESS statistic integrates the variance and the bias of the model. The retained model is the one with the smallest PRESS statistic.

RESULTS

Comparison of hydrolytic and esterifying activities from *P. fragi* cells grown at different temperatures. Several incubation temperatures ranging from 12 to 30°C were assayed to evaluate their effect on the production of the hydrolytic and esterifying activities of *P. fragi*. For each fermentation, 200 or 400 ml of a whey-based medium supplemented with 0.1% butyric acid and 0.2% food-grade ethanol was sampled regularly in order to measure the hydrolytic and esterifying activities. The production of both activities by *P. fragi* cultivated at various temperatures was measured (Fig. 1). Under such conditions, the highest amount of *p*-nitrophenol released (i.e., hydrolytic activity) occurred when the microorganism was cultivated between 24°C (149.2 U/liter) and 27°C (133.8 U/liter). The maximal hydrolytic activities detected at the other temperatures, i.e., 12 to 21°C, ranged from 39.6 to 53.5 U/liter. The esterifying activity was produced at a temperature lower than the hydrolytic activity. The highest esterifying activity, as measured during biotransformation of valeric acid and ethanol, was obtained when the microorganism was cultivated at 15°C. After 6 h of biotransformation, *P. fragi* was able to produce 0.75 μ mol of ethyl valerate per min per liter of reaction volume and for 25 g (wet weight) of cells per liter. When fermentations were run at 12 and 18°C, the esterifying activities produced were 0.47 and 0.37 U/liter, respectively. The production of esterifying activity appeared to be more affected by the incubation temperature than was the hydrolytic activity. The esterifying activity produced at 24°C (0.09 U/liter) was much lower than that at 15°C (0.75 U/liter). When the cultures were grown at 27 and 30°C, very low activity and no activity, respectively, were detected.

As expected, the fermentation time was found to be dependent on the incubation temperature, and when the incubation temperature was lowered, the maximum of both activities was reached later. For instance, the highest esterifying activity observed at 12°C was measured after 72 h of fermentation, whereas at 15°C the optimum was reached 24 h earlier. The maximal hydrolytic activity was reached at between 50 and 60 h for the cultures grown at 12, 15, 18, and 21°C. The incubation time decreased at 24 and 27°C, temperatures at which the hydrolytic production levels were the highest after 36 and 44 h, respectively. Figure 2 shows the production of maximal hydrolytic and esterifying activities, as estimated at various fermentation temperatures, and demonstrates clearly that the esterifying and hydrolytic activities occurred at two distinct incubation temperatures. The highest hydrolytic activity was produced at 24°C, while the maximal esterifying activity was obtained at 15°C.

Thermostability of hydrolytic and esterifying activities of *P. fragi* grown on whey at 15 and 24°C. Cultures were grown at 24°C for 36 h and at 15°C for 48 h, i.e., temperatures and incubation times used to obtain the maximal production of hydrolytic and esterifying activities, respectively.

Once the cells were harvested and washed, 25% bacterial suspensions were prepared in buffer as described in Materials and Methods. Initial hydrolytic and esterifying activities

FIG. 1. Time course of the production of hydrolytic (▲) and esterifying (●) activities from *P. fragi* grown on a whey-based medium supplemented with 0.1% butyric acid and 0.2% food-grade ethanol. Each activity value is the mean $\pm$ the standard deviation of three (esterifying activity) or seven (hydrolytic activity) independent experiments. Both activities are expressed in enzymatic units per liter of reaction mixture and for 25 g (wet weight) of cells per liter. No esterifying activity was detected at 30°C.

(100%) were estimated just after the preparation of the 25% cell suspensions; the suspensions were then incubated at 15 and 24°C for 4, 21, or 45 h. When *P. fragi* was grown at 15°C, the hydrolytic activity was apparently not affected by the temperature (Fig. 3). The esterifying activity of *P. fragi* grown at 15°C was less stable than the hydrolytic activity. After 21 h, the losses of esterifying activity were ca. 35% and more than 50% for cells incubated at 15 and 24°C, respectively. However, after 45 h, the loss of esterifying activity was only 55% at 15°C, whereas it was more than 90% at 24°C.

Figure 4 demonstrates that the thermostabilities of hydrolytic and esterifying activities of *P. fragi* grown at 24°C are lower than those of cells grown at 15°C. The hydrolytic activity of cells incubated at 24°C dropped at a faster rate than the

activity of the cells incubated at 15°C. Approximately 85 and 60% of the initial activities were still seen with cells incubated at 15 and 24°C, respectively, after 45 h. As for the esterifying activity, less than 40% of the initial activity was detected after the first 4 h when the cells were incubated at 24°C. After 45 h, 100% of the esterifying activity was degraded at 24°C. The esterifying activity was found to be a little more stable when the cells were incubated at 15°C. After 21 h, the loss of esterifying activity was only 40%.

Statistical models for the prediction of hydrolytic and esterifying activities. The times at which the samples were taken during the fermentation varied according to the temperature because, as the latter increased, the hydrolytic and esterifying activities reached a plateau faster.

FIG. 2. Comparison of the maximal productions of hydrolytic (▲) and esterifying (●) activities from *P. fragi* cultivated on whey-based medium at different temperatures. Each activity value is the mean $\pm$ the standard deviation of three (esterifying activity) or seven (hydrolytic activity) independent experiments. Both activities are expressed in enzymatic units per liter of reaction mixture and for 25 g (wet weight) of cells per liter. No esterifying activity was detected at 30°C.

The first fitted model used the raw, i.e., untransformed, values. Nevertheless, the verification of these assumptions revealed that there was a tendency for the variance to increase with time. A variance-stabilizing transformation, the base 10 logarithm of the activity, was applied. The modified second-order polynomial model was expressed as follows:

$$\hat{Y} = \log_{10} Y = b_0 + b_1 T + b_{11} T^2 + b_2 t + b_{22} t^2 + b_{12} Tt + \varepsilon$$

The residual analysis showed that some terms, possibly quadratic terms, were missing from the model. Quadratic interaction terms and cubic terms for main effects were added, and the fit of the model was considerably improved.

(i) Hydrolytic activity. The analysis of variance allowed assessment of the effects of the fermentation temperature, the time, and the interaction of these two factors. According to the analysis performed on the transformed hydrolytic activities, both temperature and time have an effect on the response. Furthermore, the interaction between these two factors was also found to be significant ($P < 0.0001$), indicating that the

FIG. 3. Inactivation curves of hydrolytic (▲ and △) and esterifying (○ and ●) activities from *P. fragi* grown on a whey-based medium supplemented with 0.1% butyric acid and 0.2% food-grade ethanol at 15°C (△ and ○) and 24°C (▲ and ●). Cells were produced at 15°C for 48 h. Each activity value is the mean $\pm$ the standard deviation of two independent experiments. The activity was calculated relative to the activity measured immediately after the end of the fermentation (100%).

FIG. 4. Inactivation curves of hydrolytic (△ and ▲) and esterifying (○ and ●) activities from *P. fragi* grown on a whey-based medium supplemented with 0.1% butyric acid and 0.2% food-grade ethanol at 15°C (△ and ○) and 24°C (▲ and ●). Cells were produced at 24°C for 36 h. Each activity value is the mean $\pm$ the standard deviation of three independent experiments. The relative activity was calculated from the activity measured immediately after the end of the fermentation (100%).

effect of time on the production of hydrolytic activity depends on the temperature. In other words, these results confirm that temperature and time are interdependent with respect to hydrolytic activity.

A regression model (equation 1) was estimated by using the PRESS statistic. The PRESS statistic was used to predict the production of hydrolytic activity as a function of time and temperature, and this relationship could be written as follows:

$$\begin{aligned} \hat{Y} = \log_{10} Y = & 7.321319 - 1.242633T + 0.064589T^2 - \\ & 0.001032T^3 + 0.095535t - 0.001709t^2 + 0.00000555t^3 - \\ & 0.002257Tt + 0.000072408Tt^2 + 0.000080316T^2t + \\ & 0.000002452T^2t^2 \end{aligned} \quad (1)$$

Figure 5 shows the predicted hydrolytic production dependent on the fermentation time and temperature and suggests

FIG. 5. Predicted production of hydrolytic activity as a function of temperature and time when *P. fragi* was grown on a whey-based medium supplemented with 0.1% butyric acid and 0.2% food-grade ethanol (based on equation 1). The hydrolytic activity is expressed in enzymatic units per liter of reaction mixture and for 25 g (wet weight) of cells per liter.

FIG. 6. Predicted production of esterifying activity as a function of temperature and time when *P. fragi* was grown on a whey-based medium supplemented with 0.1% butyric acid and 0.2% food-grade ethanol (based on equation 2). No esterifying activity was produced at 30°C. The esterifying activity is expressed in enzymatic units per liter of reaction mixture from cells grown at 15°C (54%) and for 25 g (wet weight) of cells per liter.

that the highest hydrolytic activity was produced at between 24 and 27°C. The error of the prediction has two components represented by the variability of the data used to define the model and the model itself.

(ii) **Esterifying activity.** Because of the imbalance in the data, PROC MIXED could not be used to estimate the effects of temperature and time and their interaction. A regression analysis on the transformed esterifying activities established that time and growth temperature were interdependent.

Equation 2 allowed the prediction of esterifying activity production as a function of the time and temperature of *P. fragi* growth as follows:

$$\hat{Y} = \log_{10} Y = -13.309602 + 0.794819T - 0.012816T^2 + 0.433058t - 0.004241t^2 + 0.000016073t^3 - 0.008285Tt \quad (2)$$

As illustrated by Fig. 6, the predicted esterifying activity was enhanced when the fermentations were done at temperatures ranging between 12 and 15°C.

Effect of enzymatic assay temperature on *P. fragi* hydrolytic and esterifying activities. The effect of the assay temperature on hydrolytic and esterifying activities was evaluated. Fermentations were therefore performed for 48 h at the two temperatures that allowed the maximal enzymatic productions, i.e., at 24°C for hydrolytic activity and at 15°C for esterifying activity. In order to estimate exclusively the effect of temperature on hydrolytic and esterifying activities, the cells used for the two tests were washed in the same buffer. Sodium phosphate buffer (0.1 M, pH 7.0) was selected since *p*-nitrophenyl valerate hydrolysis is spontaneous at alkaline pH. Enzymatic tests were performed at 37 and 12°C, which are reportedly the optimal temperatures for the determination of hydrolytic and esterifying activities, respectively (9, 22). Moreover, hydrolytic and esterifying reactions were assayed at 24 and 15°C, temperatures corresponding to the temperatures producing the highest activities for both enzymes during fermentation. The highest hydrolytic activity was obtained when the assay was performed at 37°C and was dependent on the incubation temperature for the production of *P. fragi* cells (Table 1). When the reaction was done at 24°C (84%) instead of at 37°C (100%), the relative

TABLE 1. Effect of assay temperature on hydrolytic activity^a

<i>P. fragi</i> resting cells	Hydrolytic assay temp (°C)	Hydrolytic activity (U/liter)	Relative activity (%)
Cells grown at 15°C for 48 h	12	23.0 ± 2.0	21
	15	15.0 ± 2.0	14
	24	58.5 ± 4.5	54
	37	108.0 ± 4.0	100
Cells grown at 24°C for 48 h	12	42.5 ± 1.5	20
	15	46.0 ± 0.0	22
	24	178.5 ± 3.5	84
	37	212.5 ± 4.5	100

^a The hydrolytic activity was determined with resting cells of *P. fragi* grown on a whey-based medium (two independent experiments). The enzymatic tests were carried out with sodium phosphate buffer (0.1 M, pH 7.0). Two independent assays were performed to evaluate the hydrolytic activity. The activity was calculated relative to the activity measured at 37°C, i.e., the assay temperature reported for the hydrolytic activity (22).

hydrolytic activity was slightly decreased by using *P. fragi* cells grown at 24°C, whereas the ester hydrolysis was twofold lower with cells grown at 15°C (54%). As for the esterifying activity, low assay temperatures favored the highest ethyl valerate synthesis (Table 2). When the reaction occurred with cells grown at 15°C, the ester concentration detected at 15°C was not significantly higher than that detected at 12°C ($P > 0.05$).

DISCUSSION

The objective of the present study was to determine the effect of growth temperature on the production of hydrolytic and esterifying activities of *P. fragi*. Gügi et al. (4) and Mérieau et al. (12) have reported that the activities of *P. fluorescens* MF0 extracellular proteases and lipases were expressed to a greater extent at incubation temperature (17.5°C) that was lower than the temperature used for biomass production (30°C). The protein synthesis of *P. fragi* was found to be strongly influenced by the fermentation temperature, and the proteins affected were classified into five groups (6).

Our results established that the incubation temperature has different effects on the production of the esterifying and hydrolytic activities of *P. fragi*. The hydrolytic activity was highest

TABLE 2. Effect of assay temperature on esterifying activity^a

<i>P. fragi</i> resting cells	Esterifying assay temp (°C)	Esterifying activity (U/liter, 10 ⁻³)	Relative activity (%)
Cells grown at 15°C for 48 h	12	47.0 ± 20.4	100
	15	61.5 ± 14.7	131
	24	12.3 ± 7.1	26
	37	0	0
Cells grown at 24°C for 48 h	12	40.3 ± 24.2	100
	15	26.5 ± 8.9	66
	24	17.8 ± 3.3	44
	37	0	0

^a The culture conditions are as described in Table 1. The enzymatic assays were performed with sodium phosphate buffer (0.1 M, pH 7.0) instead of Tris-HCl buffer (0.1 M, pH 9.0) in order to estimate the effect of temperature only. The amount of ethyl valerate produced was calculated after 6 h of biotransformation. Two to four independent assays were performed to evaluate the esterifying activity. The activity was calculated relative to the activity measured at 12°C, i.e., the reference assay temperature reported for the esterifying activity (9, 14, 18).

when *P. fragi* was grown at between 24 and 27°C, whereas fermentation at 15°C enhanced fatty acid ethyl ester formation. Both of these temperatures are lower than the reported optimal growth temperature of this microorganism, i.e., 30°C. Morgan (13) had already reported the existence of two biological mechanisms involved in ethyl ester synthesis by resting cells of *P. fragi*. First, a lipase should catalyze the hydrolysis of triglycerides of milk into free fatty acids. Then, an esterase should use the released fatty acids with ethanol as supplied substrate to synthesize ethyl esters. Nevertheless, the optimal fermentation temperature required to produce both activities had not been identified. Our results and the statistical analysis reported here show that the highest production of esterifying activity occurred at 12 to 15°C, while 24 to 27°C would be the appropriate temperatures to produce the highest level of hydrolytic activity. To measure the hydrolytic activity, it was found to be better to perform the assays at 37°C, whereas the esterifying activity was greatly increased at low temperatures (12 to 15°C).

A potential biotechnological application of these findings is to perform a first fermentation at 24°C for 36 h to grow *P. fragi* cells and to hydrolyze triglycerides. Then, the temperature in the tank could be lowered to 15°C to perform the ester synthesis by supplementing the medium with food-grade ethanol and *P. fragi* resting cells previously grown at 15°C for 48 h.

The highest standard deviations were obtained at 24 to 27°C and at 15°C for the hydrolytic and esterifying activities, respectively. In fact, Hébraud et al. (6) classified the *P. fragi* proteins that were affected by the incubation temperature into five classes. Class 1 contained most of the proteins whose relative synthesis was unaffected by the growth temperature. Class 2 included proteins with the highest levels of production at low temperatures (4 or 10°C). Class 3 contained peptides that were overexpressed at intermediate temperatures (15 and/or 20°C). Class 4 contained proteins synthesized at temperatures close to the optimal growth temperature, i.e., 28 to 30°C. Class 5 included proteins with the highest production levels at the supraoptimal growth temperature (34°C). The hydrolytic activity of *P. fragi* CRDA 037 could belong to class 4, which encompasses proteins produced mostly at a temperature close to the optimal growth temperature. Consequently, 27°C and, above all, 24°C may represent the critical temperature areas for *P. fragi* to produce hydrolytic activity. The esterifying activity could be included in class 3, in which protein synthesis is increased when *P. fragi* is cultivated at between 15 and 20°C. Nevertheless, 15°C is a "threshold" temperature and is probably more critical for enzyme production because it is the lower temperature limit of class 3.

Our results clearly show that the hydrolytic activity is maximally produced at 24°C, while the maximal production of the esterifying activity occurs at 15°C. However, these results raise the following question. Is the difference in the maximal-production temperatures of the two activities due to different thermostabilities of the two activities? In other words, does *P. fragi* produce less hydrolytic activity at 15°C because this activity is less stable at 15°C, and does *P. fragi* produce less esterifying activity at 24°C because this activity is less stable at 24°C?

This hypothesis was tested by first growing the cells of *P. fragi* at 15 and 24°C, temperatures corresponding to the maximal production of esterifying and hydrolytic activities, respectively. These cells were grown at 15°C (Fig. 3) and 24°C (Fig. 4); incubated for 4, 21, and 45 h at 15°C and 24°C; and finally assayed for hydrolytic and esterifying activities at 37 and 12°C. Under all of the conditions tested, the hydrolytic activity was determined to be more stable than the esterifying activity (Fig. 3 and 4).

Interestingly, although the temperature for the maximal production of hydrolytic activity was 24°C, this activity was more stable at 15°C. Thus, *P. fragi* does not produce less hydrolytic activity at 15°C because this activity is less stable at 15°C.

As for the esterifying activity, it is very much affected by the incubation temperature. When the cells were grown at 15°C, the activity did not drop during the first 4 h at 15°C (Fig. 3), whereas when the cultures were grown at 24°C the activity dropped quickly after 4 h at 24°C (Fig. 4). Thus, the esterifying activity from *P. fragi* is not produced more at 15°C than at 24°C, but the esterifying activity is quite sensitive to an incubation temperature of more than 15°C. This might explain why less activity was detected at 24°C. Furthermore, the esterifying activity was rapidly inactivated independently of the temperature of the production (15 or 24°C) and/or of the temperature used to assess the stability (15 or 24°C) after 21 h of incubation. Although 15°C corresponded to the temperature for maximal production of the esterifying activity, rapid inactivation of the activity was also observed at 15°C. In fact, the curve of inactivation of the esterifying activity of *P. fragi* grown at 15°C and assayed for thermostability at 15°C parallels the curve of inactivation of the esterifying activity of *P. fragi* grown at 15°C and assayed for thermostability at 24°C (Fig. 3). Based on these results, the assumption that *P. fragi* would produce less esterifying activity at 24°C because it is less stable at 24°C might be true.

Our results regarding the temperature stability of the hydrolytic and esterifying activities of *P. fragi* lead to additional speculation as to whether these two activities are due to one, two, or several independent proteins. Further research is needed to determine whether hydrolytic and esterifying activities that are affected differently by the fermentation temperature are due to more than one protein, as suggested by our results on thermostability and those reported by Hébraud et al. (6).

To understand how the incubation temperature affects ester production, a synthetic medium could be developed. Hellio et al. (7) demonstrated that the proteolytic activity of *P. fluorescens* MF0 grown on a minimal medium was improved after the addition of several inducers (amino acids and peptides). Furthermore, the proteolytic activity was controlled by the nature of the components but also, above all, by the growth temperature. Indeed, the optimal protease activity was independent of the inducer employed and was always expressed at 17.5°C.

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