Response Surface Models To Describe the Effects of Temperature, pH, and Ethanol Concentration on Growth Kinetics and Fermentation End Products of a *Pectinatus* sp.

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Growth curve data which had been fitted by use of the Gompertz and logistic functions have permitted the development of mathematical models to describe the growth of a *Pectinatus* sp. by several variables, namely, temperature, pH, and ethanol concentration. The activation energy of this microorganism was lower at 26 to 35°C than at 15 to 22°C. On the basis of the Arrhenius law, growth rate, maximum population density, and cell yield models have been developed by introducing the different activation energy (E_a) values. According to the model, optimal conditions were 35°C, pH 6.5, and 0% (vol/vol) ethanol for the growth rate. For cell density and cell yield, optimal conditions were 32°C, pH 6.0, and 1% (vol/vol) ethanol. No growth was observed for ethanol concentrations above 8% and pH values below 4.0. Other equations have also been made to describe the major end products fermented during fermentation by a *Pectinatus* sp. The synthesis of propionate and acetate is maximal at 28°C at pHs of 5.5 and 6.25, respectively. This model completes the model suggested by Membré and Tholozan (J. Appl. Bacteriol. 77:456–460, 1994), which includes only one variable, i.e., the glucose concentration.

The quality and safety of brewery products depend on the microbial flora which grows during production and storage. The most common causes of beer spoilage are the lactic acid bacteria such as *Lactobacillus* and *Pediococcus* spp. However, other bacteria like *Pectinatus* spp. are encountered more and more frequently. A *Pectinatus* species is an anaerobic gram-negative bacterium. This organism is nonpathogenic but induces unpleasant off-flavors due to hydrogen sulfide, fatty acids, and other volatile compounds. Propionate and acetate are the major fermentation products (7). The metabolic pathway for propionate excretion is the succinate pathway (26). *Pectinatus* species first appeared in the United States in 1972. *Pectinatus cerevisiiphilus* type strain was described by Lee et al. (11) in 1978 in the United States. Later, this microorganism was encountered in other countries, including Germany (1), Sweden (8), Norway (10), Finland (7), Japan (25), and France (23). In 1981, this bacterium represented only 1% of beer contamination, and in 1987, it represented 7%. This organism has been isolated only from the beer environment. We can therefore suppose that *Pectinatus* growth physiology really fits the beer environment. Some authors have mentioned optimal conditions of growth, but they seem to diverge. These differences can be attributed to interactions between the factors which influence the growth of *Pectinatus* species. To compensate for this problem, it is possible to use the factorial design and mathematical models which have been developed recently (22). These models can be applied to determine the growth rate (i.e., the Gompertz [6], logistic [18], Richards [17], Stanard et al. [24], and Schnute [19] models) or to estimate the

relative impact of the environmental factors on the microbial growth and, thus, to predict the risks of contamination (4, 15, 16, 21). The quadratic polynomial models were often used (2, 3).

Many factors inherent to the brewing process affect the growth of microorganisms. Temperature, ethanol concentration, and pH are the most important ones. This work suggests response curve models taking into account the main environmental factors and the interactions between factors to describe growth physiological properties of a *Pectinatus* species in relation to the fermentation products.

MATERIALS AND METHODS

Microorganism and media. *Pectinatus* sp. strain DSM 20465 was used. This strain was purchased from the Deutsch Sammlung von Mikro-organismen und Zellkulturen (Braunschweig, Germany). In all of the experiments, the inocula were prepared by culture in Man-Rogosa-Sharpe (MRS) medium at pH 6, 32°C, without ethanol for 15 h. A modified MRS medium (27) was used for all growth kinetics determinations. The initial pH values were adjusted with 1 M NaOH or 1 M H₂SO₄. The medium was dispensed in flasks flushed continuously with oxygen-free N₂-CO₂ (85:15 [vol/vol]), and Na₂S₂O₉·9H₂O (4 g liter⁻¹), and resazurin (0.5 ml of a 5% stock solution) were added just before the flasks were sealed. After sterilization (105°C for 30 min), the pH values were controlled and readjusted if necessary. For different pH values, ethanol was introduced to obtain final concentrations of 0, 2.5, and 5% (vol/vol).

Factorial design. A factorial design was used to determine the effects and interactions of temperature. The growth kinetics were determined for different temperatures (8, 15, 22, 26, 32, and 35°C), initial pH values (4, 5.3, 6, and 7.2), and ethanol concentrations (0, 2.5, and 5% [vol/vol]). For each pH and each ethanol concentration, the growth kinetics were replicated for the temperatures 26, 32, and 35°C.

Culture techniques. The bacterial cultures were done directly in disposable optical cuvettes. Sterilization of the cuvettes was achieved by dipping them in alcohol (1 day; 95% ethanol). They were dried under a flow of sterile air and left for 2 days at 60°C. The cuvettes were filled with 1 ml of the corresponding media at different pH values and ethanol concentrations in an anaerobic chamber. They were then inoculated with 10⁶ bacteria per ml with the preculture and closed hermetically with a stopper. The cuvettes were incubated in a small anaerobic incubator under oxygen-free N₂-CO₂ at different temperatures. The optical density of each cuvette was read every 4 h at 630 nm. This technique allowed the simultaneous monitoring of more than 200 growth kinetics.

Curve fitting. Computed growth curves were generated from the experimental data by use of nonlinear least-squares analysis. The nonlinear models used were the Gompertz and logistic functions, i.e., respectively, $\ln(N/N_0) =$

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$a \cdot e^{-(b/c) \cdot e^{(-c \cdot t)}}$ and $\ln(N/N_0) = a/[1 + b \cdot e^{(-c \cdot t)}]$, where N_0 is the initial number of microorganisms and N is the number of microorganisms after time (t). Corresponding growth parameters (a , b , and c) were determined by use of the STAT-ITCF computer program (Institut Technique des Céréales et des Fourrages, Paris, France). The Gompertz and logistic parameter values were subsequently used to calculate exponential growth rates and maximum population densities (28).

HPLC analysis. Carbohydrates and fermentation product analysis was carried out by high-performance liquid chromatography (HPLC; Kontron) with an OAKC column (Merck), fitted with a refractive index detector (Kontron model 475).

Statistical analysis. The logistic parameter values and the concentrations of propionate and acetate were used for the response surface analysis with quadratic polynomial models, taking into account temperature (T variable), pH (P variable), and ethanol concentration (E variable). The statistical analyses were performed by use of STAT-ITCF. The stepwise regression analysis was used. The models have been built with the untransformed parameters and with the transformed parameters, i.e., $\ln(\text{growth rate} + 1)$ and $\ln(\text{maximum population densities} + 1)$.

RESULTS

Complete factorial designs may be used to evaluate the relative significance of several factors and their complex interactions. In our study, the environmental conditions were chosen to observe a wide range of growth rates and final biomass concentrations, thus allowing good fittings of the models. Therefore, 72 variable combinations were studied in duplicate except at temperatures of 22 and 15°C.

Preliminary studies showed that the relationship between optical density and number of cells was constant during growth and given by $N = 1.6 \times 10^6 \times \text{optical density}$, with $r^2 = 0.98$. In addition, the duration of the apparent lag phase was unaffected by the initial inoculum at a size between 10^3 to 10^6 /ml (results not shown).

To determine maximal growth rates and maximal population densities, each bacterial growth curve was computed from the experimental data by use of the Gompertz and logistic functions. The logistic and Gompertz models were compared with the F test. No significant difference was observed between the two models. The growth rates and maximal population densities are reported in Table 1.

The Arrhenius equation, i.e., $\ln(\mu) = -(E_a/R) \times (1/T) + B$, where μ is the specific growth rate, E_a is the activation energy, R is the gas constant, T is the absolute temperature, and B is a constant, was used to investigate the effects of the various environmental parameters on growth rates. As shown in Fig. 1, a change in the E_a was observed at 26°C. Above this temperature, the E_a was 45.8 kJ. Below 26°C, the E_a increased to 282.8 kJ.

Growth yields, i.e., $Y = \Delta X/\Delta S$, where X is the cell biomass produced, S is the substrate (glucose and citrate) consumed, and Y is the growth yield, were calculated from maximum bacterial growth.

Modeling of growth was done by use of a response surface analysis of growth rates, maximum bacterial densities, and cell yields, taking into account temperature, pH, and ethanol concentration. Different equations were calculated and are reported in Table 2. T is the temperature in kelvins, P is the pH, and E is the ethanol concentration in percent (vol/vol). In the expression $e^{a \cdot e^{(E_a/RT)}}$, E_a is 45.8 kJ and a is 78.5 if T is superior or equal to 299 K and E_a is 282.8 kJ and a is 16.28 if T is inferior to 299 K. When the Arrhenius law was introduced into the equations, the r^2 and F test were higher. On the other hand, we have not observed any improvement for the usual transformation $[\ln(\mu + 1)]$. As for growth rates, the maximum population densities and cell yields (Table 2, equations 1, 2, and 3) were better modeled with the Arrhenius law. The Cochran test showed that residuals were normally distributed with constant variance. Figure 2 shows the good adjustment of the

model. In this model, parameter interactions were observed only with growth rates and not with bacterial densities or cell yields. The growth rate values predicted by the model were plotted at each experimental temperature. According to the model, optimal conditions were 35°C, pH 6.5, and 0% ethanol for the growth rate. For cell density and cell yield, optimal conditions were 32°C, pH 6.0, and 1% ethanol. No growth was observed at ethanol concentrations above 8% and at pH values below 4.0. A shift for growth rate optimal conditions was observed when the temperature was lowered. At a low temperature (15°C), the range for significant growth was narrowed, i.e., pH 5.0 to 6.5 and 0 to 5% (vol/vol) ethanol. At this temperature, the maximal growth rate was observed at pH 6.0 for 2.5% ethanol.

The HPLC analysis showed that under all conditions allowing growth, all sugars (11 mmol liter⁻¹) and citrate (9.5 mmol liter⁻¹) were consumed. The synthesis of propionate is higher at pH 5.5 and a temperature of 28°C. The synthesis of acetate is higher at pH 6.25 and a temperature of 26°C. The evolution of the propionate and acetate concentrations have been modeled (Table 2, equations 4 and 5) with the variables, i.e., temperature and pH.

DISCUSSION

Personal evaluation (choice of experimental points for linear regression) of the growth rate remains more or less subjective. Thus, a growth model must be used to determine accurately the maximal growth rate which is defined by the tangent in the inflection point. Among others, the Gompertz and logistic models are commonly applied to determine the lag phase, growth rate, generation time, and maximum population density (2, 3, 5, 22).

Unlike Membre and Tholozan (13), we have not observed any lag phase. To model, we used the logarithm of the number of organisms $[\ln(N/N_0)]$. For a majority of authors and for different strains, the Gompertz model gives better results than the logistic model does (5, 28). For *Pectinatus* species, an insignificant difference was observed between the two models. Around the inflection point, the curves computed from the logistic model were more similar to experimental growth kinetics than those computed with the Gompertz model. Thus, the logistic model was used for further study.

When the Arrhenius law was applied, the apparent activation energy of the *Pectinatus* species was lower (45.8 kJ) than that of other microorganisms (54 to 67 kJ). However, at temperatures below 26°C, this value increased greatly (almost six times). In aerobic bacteria, only a twofold increase was generally reported (14). This suggests that a metabolic shift (i.e., drastic changes in the rate-controlling reactions or in metabolic regulation) occurs in the *Pectinatus* species to save energy. In fact, this has to be related to the metabolic activities which have similar maximal values at two temperatures, one at 35°C and one at 22°C. The maximal specific activities, i.e., 0.194 g of substrate carbon · g of cell⁻¹ · h⁻¹, are almost five- to eight-fold lower than those in aerobic bacteria. In the same manner, cell yield, 2.09 g of dry cell · g of substrate carbon⁻¹, is twofold higher than it is in aerobic bacteria.

Quadratic polynomial models were used to predict growth parameters of the *Pectinatus* species by environmental conditions. For the growth rate, the most significant models were obtained when introducing activation energies, E_a s. In all of the recent publications, the authors show that the logarithmic transformation improves the equation from a statistical point of view. In the same way, in this study, the $\ln(\mu + 1)$ transformation also improves the model, but some deviations from

TABLE 1. Growth rates, maximum population densities, and propionate and acetate production for a *Pectinatus* species in modified MRS medium under various combinations of temperature, pH, and ethanol concentration^a

Temp (°C)	pH	Ethanol (% [vol/vol])	Growth rate	Maximum population density, 10 ⁸	Propionate (mmol/liter)	Acetate (mmol/liter)
35	5.3	0	0.2044	3.210	11.45	0
	5.3	0	0.2153	3.171		
	5.3	2.5	0.1897	3.373	18.43	14.16
	5.3	2.5	0.2081	3.229		
	5.3	5	0.1206	2.378	16.78	12.98
	5.3	5	0.1396	2.086		
	6	0	0.2952	3.514	15.77	45.66
	6	0	0.2984	3.400		
	6	2.5	0.2578	3.509	18.04	48.56
	6	2.5	0.2984	3.579		
	6	5	0.1566	2.594	13.56	43.45
	6	5	0.1890	2.661		
	7.2	0	0.2965	2.481	17.50	46.06
	7.2	0	0.2772	2.665		
	7.2	2.5	0.2145	2.438	16.24	41.43
	7.2	2.5	0.2265	2.563		
32	7.2	5	0.0991	1.022	10.66	43.10
	7.2	5	0.1071	1.307		
	5.3	0	0.2009	3.084	34.32	44.00
	5.3	0	0.1673	3.165		
	5.3	2.5	0.1882	3.382	29.81	37.15
	5.3	2.5	0.1636	3.046		
	5.3	5	0.1271	2.478	29.89	39.33
	5.3	5	0.1125	2.117		
	6	0	0.2691	3.691	19.13	56.66
	6	0	0.2537	3.614		
	6	2.5	0.2818	3.639	20.24	55.83
	6	2.5	0.2269	3.542		
	6	5	0.1719	2.746	18.28	55.70
	6	5	0.1570	2.803		
	7.2	0	0.2239	2.554	12.47	38.33
	7.2	0	0.2181	2.649		
26	7.2	2.5	0.1851	2.454	14.35	38.30
	7.2	2.5	0.1865	2.510		
	7.2	5	0.1060	0.922	8.47	35.71
	7.2	5	0.0935	0.998		
	5.3	0	0.0857	2.977	34.90	49.28
	5.3	0	0.1365	2.965		
	5.3	2.5	0.0768	2.769	32.55	46.66
	5.3	2.5	0.1130	2.660		
	5.3	5	0.1264	2.127	30.98	43.45
	5.3	5	0.1478	2.093		
	6	0	0.1897	3.701	22.82	65.00
	6	0	0.1092	3.682		
	6	2.5	0.1617	3.387	23.13	63.70
	6	2.5	0.1013	3.483		
	6	5	0.1218	2.487	14.28	58.93
	6	5	0.0973	2.954		
22	7.2	0	0.1530	2.369	16.24	46.06
	7.2	0	0.1465	2.683		
	7.2	2.5	0.1231	2.166	15.35	44.28
	7.2	2.5	0.1357	2.520		
	7.2	5	0.2559	0.672	13.33	46.66
	7.2	5	0.1582	0.987		
	5.3	0	0.0985	0.433	23.55	48.30
	5.3	2.5	0.0910	0.491	23.39	50.51
	5.3	5	0.0000	0.0000	24.05	49.03
	6	0	0.0394	1.033	17.93	48.80
	6	2.5	0.0794	0.400	17.51	50.13
	6	5	0.0000	0.0000	17.60	51.65
	7.2	0	0.0000	0.0000	0.11	14.58
	7.2	2.5	0.0000	0.0000	0.10	12.13
	7.2	5	0.0000	0.0000	0.55	10.30
15	5.3	0	0.0581	0.079	0	0

Continued

TABLE 1—Continued

Temp (°C)	pH	Ethanol (% [vol/vol])	Growth rate	Maximum population density, 10 ⁸	Propionate (mmol/liter)	Acetate (mmol/liter)
	5.3	2.5	0.0541	0.058	0	0
	5.3	5	0.0000	0.0000	0	0
	6	0	0.0000	0.0000	0	0
	6	2.5	0.0000	0.0000	0	0
	6	5	0.0000	0.0000	0	0
	7.2	0	0.0000	0.000	0	0
	7.2	2.5	0.0000	0.000	0	0
	7.2	5	0.0000	0.000	0	0

^a The values at 8°C and pH 4 are not shown.

experimental values were observed for the limit values, i.e., when the growth is almost nil. This can be due to the fact that the logarithmic transformation overestimates a low growth rate in the regression. On the basis of the Arrhenius law, models taking into account $1/T$ were used previously (12). Although the changes in E_a at low temperatures are well known, the influence of E_a was not investigated, even for wide temperature ranges (12). This model, i.e., equations 1, 2, and 3 (Table 2), has been successfully applied for *Pectinatus* sp. strain DSM 20465.

A comparison of the values calculated by use of the model and those from other experimental studies gives a general idea of the validity of the model. The results predicted by the model are close to those we have already found in a preliminary study, at least from the experimental point of view; i.e., there is growth until pH 4, and growth is optimum at pH 6 and at a temperature between 30 and 37°C (27). These results are partly confirmed by other authors: Haikara et al. (7) and Soberka et al. (23) have shown that there is growth until pH 4.5 and 4, respectively. The limiting conditions for the development of *Pectinatus* species in a medium containing ethanol, i.e., 4 (27), 4.5 (7, 20), 6 (23), and 8% (vol/vol) ethanol (unpublished results), are very different according to the different authors. These differences can be explained by the fact that these authors do not take into account the interactions between the variables (different pH values and temperatures).

FIG. 1. Arrhenius plot $\ln(\mu) = f(1/T)$ for a *Pectinatus* sp.

TABLE 2. Quadratic models for the effects and interactions of temperature, initial pH, and ethanol concentration on growth rates, maximum population densities, cell yield, propionate production, and acetate production for *Pectinatus* species in modified MRS medium

Equation no.	Equation
1.....	Growth rate = $-0.03535 e^{(E_a/RT)} + 0.63669 P + 0.01929 E - 1.53616 e^{(E_a/RT)} - 0.05304 P^2 - 0.00377 E^2 + 0.30083 e^{(E_a/RT)} \cdot P + 0.16096 e^{(E_a/RT)} \cdot E - 0.04578 e^{(E_a/RT)} \cdot P \cdot E - 1.91758$ (degree of freedom = 68; $r^2 = 0.96$; F test = 164.66)
2.....	Maximum population density = $41.76663 e^{(E_a/RT)} + 9.55536 P + 0.12168 E - 120.50600 e^{(E_a/RT)} - 0.79800 P^2 - 0.06206 E^2 - 28.54130$ (degree of freedom = 68; $r^2 = 0.93$; F test = 137.65)
3.....	Cell yield = $28.5900 e^{(E_a/RT)} + 6.5518 P + 0.0846 E - 82.6094 e^{(E_a/RT)} - 0.5465 P^2 - 0.0852 E^2 - 19.5827$ (degree of freedom = 68; $r^2 = 0.93$; F test = 138.24)
4.....	Propionate production = $4.1179 T + 59.5291 P - 0.1196 T^2 - 6.3525 P^2 + 0.4829 TP - 206.2117$ (degree of freedom = 71; $r^2 = 0.78$; F test = 34.20)
5.....	Acetate production = $11.9675 T + 215.1847 P - 0.3250 T^2 - 19.2442 P^2 + 0.9691 TP - 780.1397$ (degree of freedom = 71; $r^2 = 0.91$; F test = 62.05)

Indeed, equation 1 shows that a significant interaction exists between ethanol and temperature as well as between ethanol, temperature, and pH.

Unpublished results have shown that the glucose and citrate concentrations used do not limit the growth rate but do limit the biomass and the metabolized products. According to the model of Membré and Tholozan, the fermentation balance depends on the initial glucose concentration. Propionate and acetate were the major end products formed during fermentation of glucose and citrate. Considering the important proportions of acetate, which is synthesized, we can suppose that it has an important role in *Pectinatus* cell energy. The balance between the synthesis of propionate and succinate shows that the *Pectinatus* species uses the succinate pathway to synthesize propionate. Other authors (7, 26) have obtained similar results. As for *Propionibacterium acidipropionici* (9), the synthesis is characterized by an optimal pH of about 5. The optimal temperature for the synthesis of propionate is lower than the growth temperature. Unlike acetate synthesis, there is a difference between the optimal pH for propionate synthesis and that for growth. Our results show that the metabolite synthesis is not directly linked to growth. The presence of ethanol limits

the growth but does not seem to interfere significantly with the metabolism. The equations 4 and 5 (Table 2) do not take into account the ethanol concentration, but they show that there is an interaction between pH and temperature.

This model, linked to the Membré and Tholozan model, provides a better knowledge of growth and propionate and acetate production during beer spoilage. The models permit the evaluation of the risk of contamination of beer by a *Pectinatus* species in the brewery process. We can suppose that a low-temperature fermentation limits the risk of beer contamination by a *Pectinatus* species. During fermentation at higher temperatures, a *Pectinatus* species can develop, leading to beer alteration by an important synthesis of propionate and by the formation of an important cloudiness in a beer with low alcohol content. For beer during storage, the temperature can no longer be used to limit *Pectinatus* growth. In this case, only the ethanol concentration and the absence of carbon source can limit the growth and the production of propionate.

This work indicates the efficiency of both the factorial designs and the mathematical models for the study of the growth physiology of bacteria. Other results (unpublished data) obtained with *P. cerevisiophilus* and *Pectinatus frisingensis* strains suggest that the model proposed in this study is useful for the whole *Pectinatus* genus.

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FIG. 2. Relationships between the experimental values and the values calculated with equation 1.

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