Effects of Temperature, pH, and NaCl on Growth and Pectinolytic Activity of Pseudomonas marginalis

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The interaction of temperature (4, 10, 18, and 30°C), pH (6, 7, and 8), and NaCl (0, 2.5, and 5%) and their effects on specific growth rate, lag phase, and pectinolytic enzymes of Pseudomonas marginalis were evaluated. Response surface methodology was adapted to describe the response of growth parameters to environmental changes. To obtain good conditions of storage, the combined action of salt and temperature is necessary. At 4°C with an NaCl concentration of 5% and a pH of 7, the lag time was 8 days and no growth was observed at 4°C with 5% NaCl and a pH of 6. In the absence of salt, P. marginalis could grow regardless of temperature and pH. Pectate lyase and pectin lyase were produced by P. marginalis, while pectin methyl esterase activity was not observed in our culture conditions. The enzyme production depended on temperature, pH, and salt concentration but also on the age of the culture. Pectinolytic enzymes were abundantly excreted during the stationary phase, and even at 4°C, after 2 weeks of storage, enzyme activities in supernatant culture were sufficient to damage vegetables. Both bacterial growth and enzymatic production have to be taken into account in order to estimate correctly the shelf life of vegetables.

Pseudomonas marginalis is a phytopathogenic bacteria frequently reported as an important cause of spoilage of vegetables before and after harvest. Gram-negative, psychrotrophic bacteria are thought to be the predominant flora on lettuce (11, 16, 18, 19), and P. marginalis is often described as a pectinolytic bacterium (1, 14). Treatment of salad vegetables with a solution containing chlorine can be used to reduce the number of contaminating microorganisms, but the processes of cutting and mixing create conditions that favor microbial growth (2). Lettuce has a shelf life of about 2 weeks, depending upon initial quality, storage temperature, and packaging conditions. The major symptom of the bacterial soft rot is the maceration of plant tissue that results from the destruction of the middle lamella and primary cell walls in colonized tissue. The ability of soft rot bacteria to cause vegetable spoilage results mainly from their ability to produce massive amounts of pectinolytic enzymes. Pectate lyase (PL) activity has been detected previously in supernatants of phytopathogenic bacteria and has been described as the most important cause of soft rot disease (6, 7, 17, 26). Pectin lyase (PNL) and pectin methyl esterase (PME) are also cited as pectinolytic enzymes produced by microorganisms (6). P. marginalis has been described as bacteria producing PL (5, 21), PNL (22, 25), and small amounts of PME (5, 21).

To quantify growth and enzyme production of P. marginalis during storage at low temperature, and to determine the influence of pH and NaCl concentration on shelf life of a food product, we have chosen a predictive microbiology approach. This method uses mathematical equations to estimate how the growth characteristics (e.g., lag time and specific growth rate) are affected by storage conditions (3). Among the various models proposed, the response surface method is widely employed in food microbiology (4, 13). On the other hand, the Arrhenius equation is used to describe the effect of temperature on Pseudomonas fluorescens growth (1, 27), and its application is generalized when several factors act in combination (9).

MATERIALS AND METHODS

Organism, media, and culture conditions. The organism used in this study was P. marginalis CFBP 1387 (ATCC 10844, NCPPB 667). The growth medium was peptone (4 g liter⁻¹) (Unipeptone URS 150; Sanofi Bio-industries, Paris, France)-yeast extract (2 g liter⁻¹)-NH₄Cl (1 g liter⁻¹)-MgSO₄(0.5 g liter⁻¹), supplemented with piperazine-N,N’-bis(2-ethanesulfonic acid) (50 mM), pH was adjusted according to experimental design, with NaOH or H₂SO₄.

Growth experiments were done in 50-ml volumes of medium in 250-ml flasks. The inoculum was 4% (vol/vol) of a 2-day culture incubated at 30°C and pH 7, in order to obtain an initial biomass concentration of 10⁵ CFU/ml. Growth was measured with a spectrophotometer at A₅₅₀. Optical density was linearly correlated to dry weight. Bacterial growth curves were fitted by a sigmoidal growth model (modified Gompertz) with three parameters (28): ln(N/N₀) = A · exp{−exp[μ(t)·e·(λ − t)/l]}¡, where A is the asymptotic level ln(N/N₀), N is cell density, μ is the maximum specific growth rate (hours⁻¹), λ is the lag phase duration (hours), l is time (hours), and e is exponential 1 [exp(1) = 2.718].

Enzyme assays. After centrifugation (4,000 × g, 10 min), the culture supernatant was filtered through 0.45-μm-pore-size paper, stored at −20°C, and later assayed for pectinolytic enzyme activity.

PME activity measurement is based on the decrease in pH of the reaction mixture due to the increase in free carboxyl groups during enzyme action (12). Enzyme sample (0.1 ml) is added to 1.5 ml of 2% (wt/vol) peptone in 10 mM sodium phosphate buffer (pH 7) and 1 ml of distilled water. One unit is defined as the amount of enzyme which causes a decrease in pH of 0.1 in 30 min.

The cleavage of glycosidic linkages of the galacturonan chain...
occurs in the presence of alkali by a transelimination reaction which yields oligouronides with a Δ4-5 double bond in the terminal residue of the nonreducing end of the chain (subsequently referred to as unsaturated galacturonic acid). The division of lyases is carried out on the basis of their preference for highly esterified pectin acid (PNL) or pectic acid (PL) (6).

PL activity is measured by the increase in A235 of 4.5 unsaturated reaction product. The assay has been previously described (8) and was modified as follows. First, 0.3 ml of 1% (wt/vol) polygalacturonic acid neutralized by NaOH was mixed with 1.5 ml of CaCl2 solution (0.0005 M in 0.1 M Tris-HCl [pH 9]) and 1.1 ml of distilled water. Then, 0.1 ml of enzyme sample was quickly added and the increase in A235 was measured in a 3-ml cuvette with a 1-cm light path at 30°C and monitored as a function of time with a recording spectrophotometer. One unit is defined as the amount of enzyme which produces 1 μmol of unsaturated product per min. The molar extinction coefficient for the unsaturated product at 235 nm (ε235) is 4,600 M−1 cm−1, and the enzymatic unit (U) is calculated as U = [(ΔA)/ε235] · 106, where ΔA is an increase of absorbance per minute. To measure activity at various temperatures, the reaction was performed at 5.5, 11, 17.5, 25, 30, and 33.5°C.

PNL activity was measured by the reaction between the unsaturated end product of pectin degradation and thiobarbituric acid (23). After incubation of 5 ml of pectin solution (1% in 0.05 M Tris-HCl buffer [pH 8.5])–1 ml of 0.01 M CaCl2–1 ml of enzyme solution–3 ml of distilled water for 2 h at 30°C, the reaction was stopped by the addition of 0.6 ml of 9% (wt/vol) ZnSO4, 7 H2O and 0.6 ml of 0.5 M NaOH. Precipitated protein and unused substrate were removed by centrifugation (3,000 × g, 10 min). The supernatant was mixed with barbituric acid, and the mixture was heated in a boiling-water bath for 30 min. After cooling, the A450 was read against a reference cuvette containing the same reagents but which were not left to incubate. One unit is defined as the amount of enzyme which causes a change in absorbance of 0.01. The incubation was performed at 4, 10, 18, and 30°C to measure activity as a function of temperature.

Experimental design. An experimental design was employed to assess the effects of temperature, initial pH, and salt concentration of P. marginalis growth and enzyme production. The levels of factors and nature of salt were chosen following processes widely used in ready-to-use vegetables. A factorial design was used, with four temperatures (4, 10, 18, and 30°C), three pH levels (6, 7, and 8), and three NaCl concentrations (0, 2.5, and 5% [wt/vol]). Two experiments were performed at each combination of temperature, pH, and salt; the results given below are the means of the data.

Statistical analyses. Statistical analyses (regression coefficients and analysis of variance) were done by using the GLM procedure of the Statistical System Analysis (SAS Institute Inc., Cary, N.C.). Two data are missing because of lack of growth (for the culture under conditions of 4°C, pH 6, and 5% NaCl). We do not believe that the two omitted values affect model development.

RESULTS AND DISCUSSION

Growth parameters. For each combination of salt, temperature, and pH studied, no difference was observed in the final absorbance in the stationary phase, except where there was no growth. Thus, we only took into account the specific growth rate and the lag phase.

(i) Specific growth rate. The Arrhenius equation is often used to describe the relationship between growth rate and temperature (1, 4, 27). It can be employed at low tempera-

It is given by the following equation: 

$$
\ln \mu = - (E_a/R) \cdot (1/T) + C,
$$

where \( E_a \) is activation energy, \( R \) is gas constant, \( T \) is absolute temperature, and \( C \) is a constant. On the other hand, response surface methodology is widely employed to predict growth rate as a function of several factors (3, 13). In this case, linear, quadratic, and interactive effects of factors are tested in a general linear regression model. We have decided to describe the effects of the three factors in a general model which takes interactive effects of the variables into account. Response surface methodology has been performed with the factors 1/T (inverse of absolute temperature), pH, and NaCl concentration.

Only significant terms (t test; \( P < 0.05 \)) were written in the final equation. The equation of specific growth rate is written as follows: 

$$
\ln \mu = 152.04 - 93751.2 \cdot (1/T) - 1.64 \cdot \text{NaCl} + 5.49 \cdot \text{pH} + 12953197 \cdot (1/T)^2 - 0.06 \cdot \text{NaCl}^2 - 0.39 \cdot \text{pH}^2 + 204.24 \cdot (1/T) \cdot \text{NaCl} + 0.13 \cdot \text{pH} \cdot \text{NaCl}.
$$

The analysis of variance indicated that the model was significant (\( P < 0.05 \)), and had a \( R^2 \) value of 0.87. This growth model equation is similar to the Davy equation (9), but here, salt interactions with temperature and pH are taken into account. The Arrhenius equation is not completely applicable in our temperature range. The significant quadratic term 1/T2 indicates that we have studied temperature effect slightly below the optimum growth rate temperature. In Fig. 1, observed and predicted values are plotted as functions of temperature and pH at two concentrations of NaCl (0 and 2.5%).

Temperature had a strong inhibitory effect on growth rate. P. marginalis grew on synthetic culture medium at low temperature (4°C) with a specific growth rate of 0.033 h−1 (generation time of 21 h) at pH 7 and 0% NaCl, while in the same pH and salt concentration, \( \mu \) was 0.23 h−1 (generation time of 3 h) at 30°C.

In culture media without NaCl, the optimum growth rate was observed at pH 7 at all temperatures. With added salt, little or no difference was observed between the growth rate of culture grown on media at pH 7 and at pH 8, while acid culture media inhibited growth at all temperatures.

NaCl decreased the growth at all pHs and temperatures. For instance, at 18°C and pH 7, \( \mu \) fell from 0.1 h−1 (generation time of 7 h) with 0% NaCl to 0.076 h−1 (generation time of 9 h) with 2.5% NaCl and to 0.023 h−1 (generation time of 30 h) with 5% NaCl. We did not observe growth of P. marginalis after 42 days on culture medium with 5% NaCl and pH 6 at 4°C. Thus, the combination of an acid medium and added salt is a good condition to avoid food spoilage, particularly at low temperatures.

(ii) Lag time. According to the Davy approach (10), the logarithm of inverse of lag time (ln [1/L]) was modeled as a function of the three variables temperature, pH, and NaCl concentration. For specific growth rate modeling, response surface methodology was employed to elaborate a second order polynomial model. Examination of the fitted model by t test indicated that the three linear terms temperature (1/T) NaCl, and pH; one quadratic term, pH2; and two interactions, 1/T · NaCl and pH · NaCl, were significant. The equation of lag time is written as follows: 

$$
\ln (1/L) = - 0.2554 - 9499.95 \cdot (1/T) - 7.22 \cdot \text{NaCl} + 8.85 \cdot \text{pH} - 0.6114 \cdot \text{pH}^2 + 1615.8 \cdot (1/T) \cdot \text{NaCl} + 0.1336 \cdot \text{NaCl} \cdot \text{pH}.
$$

The analysis of variance indicated that the model was significant (\( P < 0.05 \)), and had a \( R^2 \) value of 0.89. In Fig. 2, observed and predicted values are plotted as functions of temperature and pH at two concentrations of NaCl (0 and 2.5%).

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Salt strongly increased lag time. For instance, at pH 6 and 30°C, L increased from 4 h with 0% NaCl to 54 h with 2.5% NaCl and 990 h (6 weeks) with 5% NaCl.

The quadratic effect of pH indicated that the shortest lag time occurred between pH 7 and 8. Temperature had a great effect on lag time.

(iii) Growth inhibition. According to King et al. (16), the bacterial population on processed lettuce was dominated by members of the genera Pseudomonas (57%), Serratia (8%), and Erwinia (8%). The majority of the bacterial isolates on salad were described as psychrotrophic (18). To get good conditions of food storage, the combined action of temperature and salt is necessary. Ready-to-use vegetables, at pH 6 or 6.1 (16), would have a shelf life of 6 days at low temperature (4°C) and a NaCl concentration of 2.5%. Without salt addition, P. marginalis grew at 4°C and pH 6 after a lag time of 20 h, with a growth rate of 0.027 h⁻¹. Under these conditions, after 2 weeks, with an initial contamination of 4.1 log₁₀ CFU/g of unsealed salad mix (16), P. marginalis population achieves until 7.8 log₁₀ CFU/g. This value concurs with the count of bacterial contaminants in lettuce stored at 2.8°C (7.3 log₁₀ CFU/g).

Pectolytic enzyme production and activity. Ceponis and Friedman (5) reported that PME activity was found to be slight in P. marginalis culture filtrates on lettuce, and according to Nasuno and Starr (21), small amounts of PME could be excreted by P. marginalis. In this study, no activity of PME was noted at any temperature, pH, or salt concentration. PL and PNL have been found in culture filtrates, and their activities were quantified. Their presence in P. marginalis had been already reported by several authors (5, 21, 22, 25), but here, the effect of environmental factors such as temperature, pH, and NaCl concentration on their production is described.

(i) Kinetics of production of PL and PNL. The production of PL and pectin lyase (PNL) quickly increased during the early stationary phase in the supernatant. As the final cell density was the same whatever combination of factors, except in the case of no growth, the amounts of enzymes were been comparable. With an initial pectin concentration of 4 g/liter, the production was still detected during the stationary growth phase. In Table 1, PL and PNL production are noted as a function of culture time and temperature. At 18°C and pH 7, without NaCl addition, P. marginalis achieved the stationary phase after 3 days of incubation; at this time PL and PNL activities were 5.8 and 7.6 U, respectively. Enzymatic production increased linearly, and in 17-day culture supernatants, great pectinolytic activities were detected (PL, 20.2 U; PNL, 12.1 U). Moreover, after 2 weeks of storage at 4°C, PL and PNL activities were as strong as 3-day culture supernatant enzyme activities at 18°C. Under these conditions, P. marginalis could be a bacterial contaminant which causes important damage to vegetables stored for more than 1 week even in a cool room.
(ii) Effect of temperature on production and activity. Two kinds of experiments were carried out to quantify the effect of temperature on pectinolytic enzyme production and activity. First, PL and PNL production was measured in supernatant of cultures incubated at various temperatures. After 3, 7, 10, 14, and 17 days of culture, pectinolytic production was determined at 30°C following the methods described above. In Table 1, we can see that after 7 days of culture at 4°C, *P. marginalis* produced 1.3 U of PL (and 4.2 U of PNL) whereas at 18°C, *P. marginalis* produced 7.6 U of PL (and 7.8 U of PNL). The storage of food products in cool rooms decreases greatly the production of pectinolytic enzymes and thus allows damage to vegetables to be avoided. The response of enzyme production at various temperatures is different for PL and PNL. With our temperature range (4 to 30°C) no optimum production was found for PL. The production increased with temperature of growth culture. However, PNL production was maximum at a temperature of 18°C. For this enzyme, the optimum production was below the optimum growth temperature (25 to 30°C). In the literature, the majority of the bacterial isolates on salad were characterized as psychrotrophs, capable of growth at both 6 and 30°C (16, 18). Thus, psychrotrophic bacteria such as *P. marginalis* are adapted to growing below their optimum temperature and can damage vegetables because of their enzymatic content. This behavior has already been observed for protease and lipase from *P. fluorescens* (15, 20). This result may be important for storage temperature. A slight increase in the temperature may increase the production of damaging enzyme without a parallel increase in the number of bacteria.

Finally, measurement of activities at various temperatures was performed. Supernatants of culture grown at 4°C and at 18°C were analyzed, and PL and PNL activities were quantified. In Fig. 3, the enzyme activities of an 11-day culture grown at 18°C are plotted as a function of temperature. PL activity increased exponentially with temperature, while PNL activity increased linearly with temperature. We obtained the same conclusion with a 33-day culture grown at 4°C. Activity was

![FIG. 2. Effect of temperature and pH on lag time. Shaded prism, observed values; pillar, predicted values. (a) NaCl concentration of 0%; (b) NaCl concentration of 2.5%.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>PL production</th>
<th>PNL production</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1.3</td>
<td>4.2</td>
</tr>
<tr>
<td>10</td>
<td>1.7</td>
<td>4.1</td>
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<tr>
<td>18</td>
<td>7.6</td>
<td>7.8</td>
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<tr>
<td>30</td>
<td>12.1</td>
<td>8.1</td>
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</tbody>
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Table 1. Production of PL and pectin lyase PNL as a function of temperature and time of incubation, for supernatant of culture grown at pH 7 without salt addition.
divided by 4 for PL, and by 2 for PNL, when the temperature fell from 18 to 4°C.

In conclusion, at low temperature (4°C) _P. marginalis_ has a weak potential to damage vegetables, but if the temperature increases to 18°C (ambient air temperature) for only a few hours, enzymes produced at low temperature will become active, and then food products will be spoiled rapidly.

(iii) Effect of pH and salt concentration. In no case was PL and PNL production observed in a culture medium with 5% NaCl. Salt addition greatly inhibited enzyme production. In Table 2, the effect of pH and NaCl concentration is given for two culture incubation temperatures. Without salt addition, PL production increased with pH. On the other hand, there was no difference between PNL production at pH 7 and that at pH 8. At pH 6, pectinolytic enzyme production was the lowest at all temperatures and salt concentrations. Many vegetable salads are acidified by the addition of vinegar-based dressings, and ready-to-use vegetables are commonly mixed, chopped, sliced, or grated; these products fall, therefore, into the low-acidity range (18). _P. marginalis_ should not be able to produce large amounts of pectinolytic enzymes under conditions of chill storage, because of the pH of lettuce and ready-to-use salads. In conclusion, the predictive microbiology approach, applied to the study of phytopathogenic microorganisms, has to take into account both bacterial growth and enzymatic production in order to give a correct indication of spoilage when estimating the shelf life of food.

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**REFERENCES**


