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Time Course of Growth and Protease Production by Spoilage Bacteria Under Different Culture Conditions

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Four strains of proteolytic bacteria which were assessed as potential fish spoilers were used for the study. They were *Pseudomonas* sp. Ca 173 isolated from *Penaeus indicus*, *Pseudomonas* sp. Ca 386 from *Metapenaeus dobsoni*, *Vibrio* sp. Ca 377 from *Liza parsia* and *Vibrio* sp. Ca 761 from *Penaeus indicus*. The influence of pH, temperature and sodium chloride concentrations on the growth and protease production of these bacterial isolates were studied. The study shows that the requirements of the normal flora of fish and prawns for maximum protease production are 30°C, neutral pH (7) and absence of NaCl. Moreover, the protease production was observed at late logarithmic or early stationary phase. This suggests that the inhibition of the bacterial growth in the late logarithmic or early stationary phase will arrest the bacterial fish spoilage to some extent.

Key words : Spoilage bacteria, growth, protease production, culture conditions.

Influence of culture conditions on growth and physiology of bacteria are wide and varied. Regarding the growth characteristics and protease formation, most of the earlier reports are on the effect of various chemicals, cations and organic components (Kato *et al.*, 1972; Sashihara *et al.*, 1975; Sakata *et al.*, 1977 and Makino *et al.*, 1981). The effect of incubation temperature on the growth of marine bacteria and protease production have also been studied (Kato *et al.*, 1972 and Makino *et al.*, 1981). The current study was initiated to gain information on the time course of growth and protease production by four bacterial isolates at four different temperatures, pH and NaCl concentrations.

Materials and Methods

Four strains of proteolytic bacteria isolated from seafoods were used for the study. They were *Pseudomonas* sp. Ca 173 isolated from *Penaeus indicus*, *Pseudomonas* sp. Ca 386 from *Metapenaeus dobsoni*, *Vibrio* sp. Ca 377 from *Liza parsia* and *Vibrio* sp. Ca 761 from *Penaeus indicus*. Nutrient broth (peptone and beef extract, 0.5 g l^{-1} each) prepared in 0.05 M Tris - HCl buffer of pH 7.6 was employed for studying the effect of temperature of incubation (30, 35, 40 & 45°C) and sodium chloride concentrations (0, 10, 20 & 30 g l^{-1}) on the growth and protease formation of the selected bacterial isolates. Likewise, for observing the influence of pH on the same physiological traits, nutrient broth of pH 6, 7, 8 and 10 were prepared in Tris - Maleic acid (pH 6), Tris - HCl (pH 7 & 8) and $NaHCO_3$ - Na_2CO_3 (pH 10) buffers of 0.05 M each. Growth was recorded by measuring the optical density at 600 nm in a Hitachi Model 200-20 spectrophotometer against uninnoculated controls.

Protease activity was assayed by a modification of the casein digestion method of Kunitz (1946). Casein (Hammerstein) (0.6%) in 0.05 M Tris - HCl buffer (pH 7.6)

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was used as the substrate. To 2 ml of the casein substrate, 1 ml of the culture supernatant (crude enzyme) was added and incubated at 30°C for 30 min. The reaction was stopped with 2.5 ml of 0.44 M Trichloroacetic acid (TCA) solution. The precipitated protein was filtered through Whatman No. 1 filter paper and the absorbance of the filtrate was measured at 280 nm against blanks to which TCA had been added before the enzyme.

Cell suspension for inoculation were prepared by harvesting 18 to 20 h old nutrient agar slant cultures in sterile physiological saline (0.85% NaCl) and adjusting the optical density to 1 at 600 nm. One ml each of the cell suspension was inoculated to 100 ml of the different media prepared.

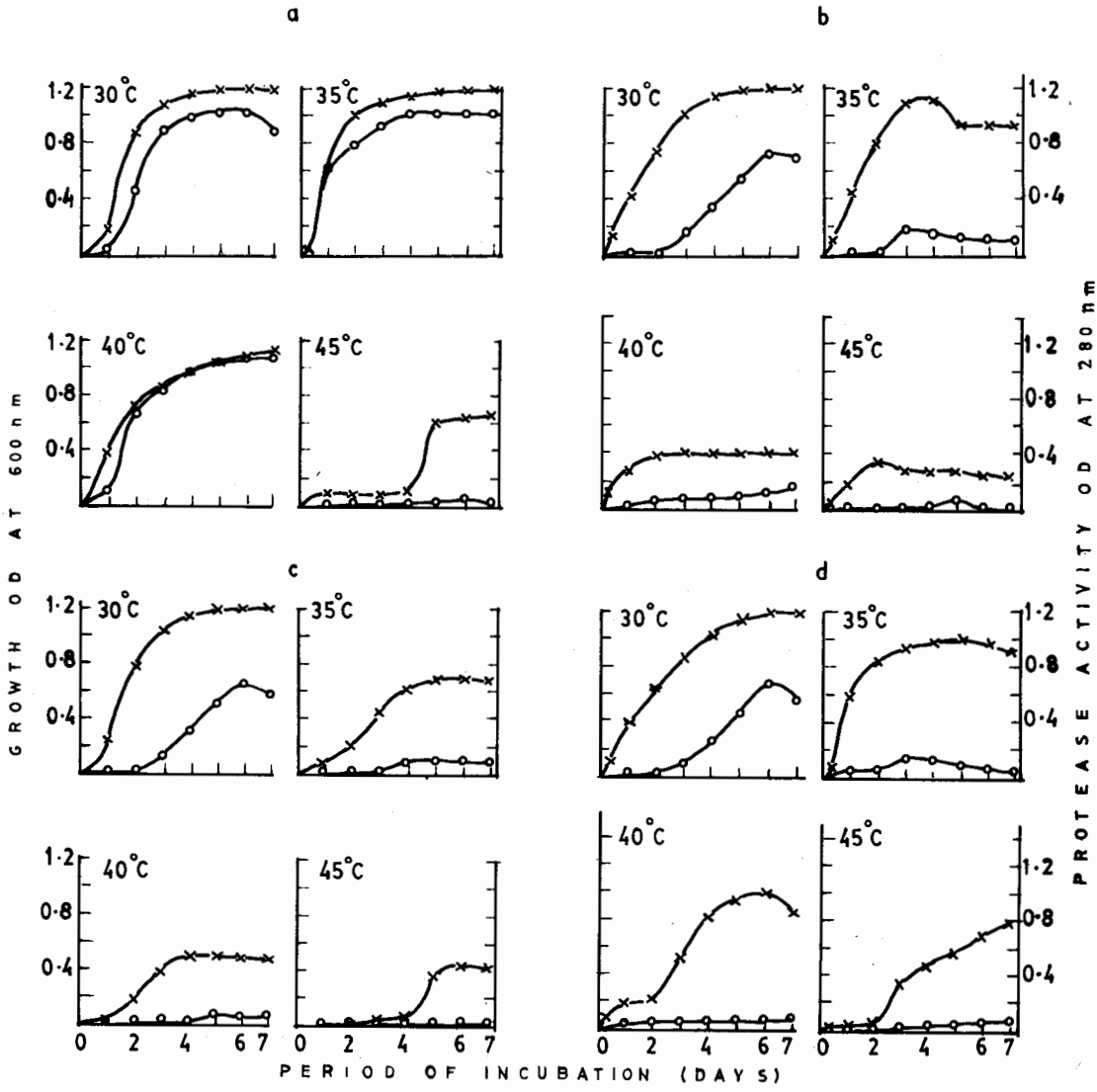

Fig. 1. Time course of growth (x — x) and protease production (o — o) at different temperatures.
a - *Pseudomonas* sp. Ca 173; b - *Pseudomonas* sp. Ca 386; c - *Vibrio* sp. Ca 377; d - *Vibrio* sp. Ca 761.

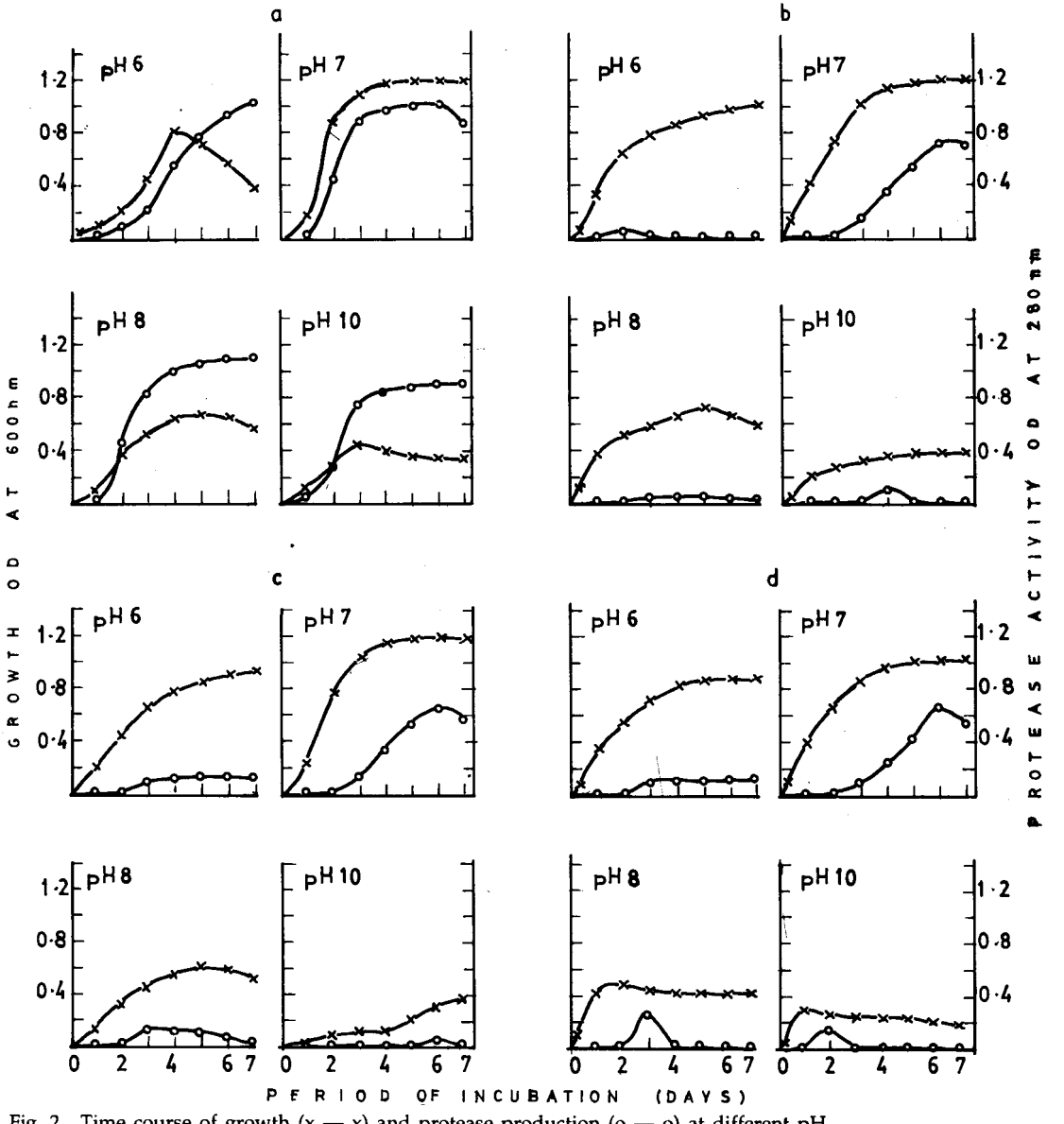

Fig. 2. Time course of growth (x — x) and protease production (o — o) at different pH.
a - *Pseudomonas* sp. Ca 173; b - *Pseudomonas* sp. Ca 386; c - *Vibrio* sp. Ca 377; d - *Vibrio* sp. Ca 761.

Immediately after inoculation (0 h) the cell density (optical density) and protease activity of the media were estimated. The culture flasks were incubated for 7 days and the growth and protease activity were measured at 24 h intervals. For estimating the protease activity, a portion of the culture broth was centrifuged at 7500 rpm for 15 min and the supernatant was taken for analysis.

Results and Discussion

At 30°C, all the four isolates could grow well and produce large quantity of protease while in other temperatures considerable growth with little protease pro-

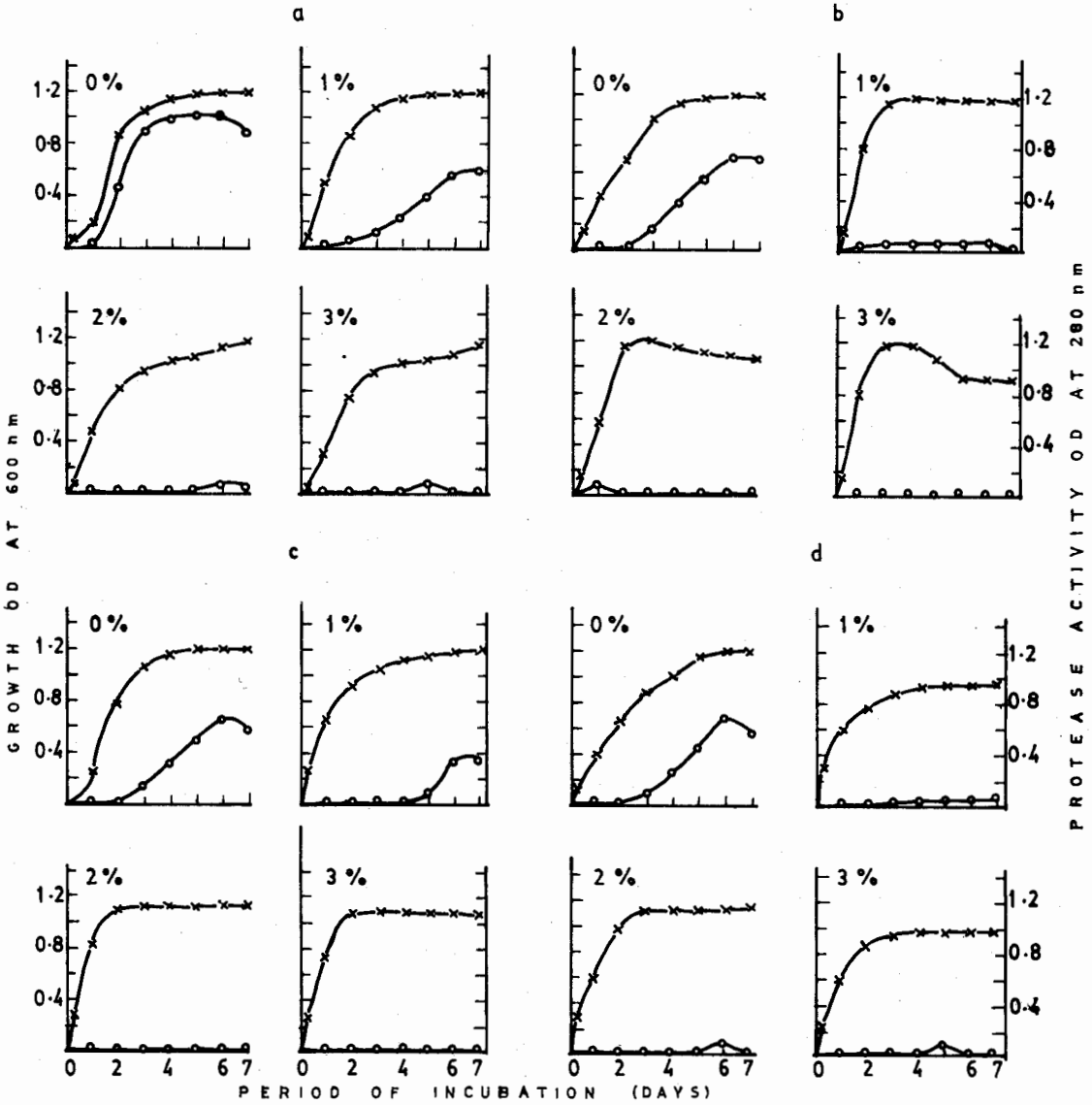

Fig. 3. Time course of growth (x — x) and protease production (o — o) at different NaCl concentrations. a - *Pseudomonas* sp Ca 173; b - *Pseudomonas* sp. Ca 386; c - *Vibrio* sp. Ca 377; d - *Vibrio* sp. Ca 761.

duction was observed (Fig. 1). However, *Pseudomonas* sp. Ca 173 exhibited significant growth and protease formation at 35 and 40°C also. Protease production commenced during the late logarithmic phase in all the cases except in *Pseudomonas* sp. ca 173 which showed protease activity in the early logarithmic phase itself.

The pH optima for growth and protease formation was found to be 7 in all the cases (Fig. 2).

Presence of NaCl in the growth medium inhibited the production of protease by all the cultures. Whenever protease production occurred, it was at late

logarithmic or stationary phase of the growth cycle except in the case of *Pseudomonas* sp. Ca 173, where the production started in the early logarithmic phase itself (Fig. 3).

The observation that the protease production starts when the culture enters late logarithmic or stationary phase is in agreement with the earlier reports (keil-Dlouha *et al.*, 1976; Robertse *et al.*, 1978; Reid *et al.*, 1980; and Long *et al*, 1981). On studying the penetration of selected strains of proteolytic and non-proteolytic bacteria into cattle meat, it was reported that penetration of meat by bacteria resulted from the break down of the connective tissue between muscle fibres (endomysium) by proteolytic enzymes secreted by the bacteria (Gill & Penney, 1977). Based on the present observation, it can be postulated that bacterial multiplication up to late logarithmic phase at the surface and in the intestine of fish and prawns is essential for the production of protease so as to achieve an active invasion into the flesh by the lysis of connective tissue. The requirement of 30-35°C for maximum protease production points to the need for maintaining the fish at low temperatures for arresting the protease production by bacteria and thereby the invasion of flesh. The observation that pH 7 is highly favourable for protease formation explains why the bacterial invasion does not occur during rigor mortis. Further, inhibition of protease formation by NaCl indicates that addition of NaCl to fish (curing) could prevent the bacterial invasion through surfaces.

The study reveals that the requirements of the representative normal flora of fish and prawns for maximum protease production are a temperature of 30°C, neutral pH (7), absence of NaCl and the entry of growth cycle into the late logarithmic or staionary phase. Reduction of temperature, alteration of pH to acidic levels and the addition of NaCl to fish can suppress bacterial growth and protease formation. A proper combination of all these will in turn result in the inhibition of bacterial invasion through the surface of seafoods.

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