Le Lait (1985), *65* (649-650), 169-183

Effect of some nutritional and environmental factors on extracellular protease production by *Pseudomonas* SP.B-25

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SUMMARY

Fortification of the 1 % tryptone basal medium with 0.25 % yeast extract resulted in 2.6-fold increase in protease production by *Pseudomonas* sp.B-25. Tryptone yeast extract in the medium could not be substituted by other equivalent media ingredients. Of the various inorganic salts incorporated in the modified basal medium (1 % tryptone + 0.25 % yeast extract), addition of 0.2 % potassium phosphate salts elicited maximum enzyme production. However, none of the fermentable sugars tried as supplements to the formulated tryptone-yeast extract-phosphate (TYEP) medium proved useful. Maximum protease production was observed in TYEP medium at pH 7.0 when inoculated with 2.5 % of 24 hr old broth culture and incubating at 22° C for 48 h on a gyratory shaker. The protease production by Pseudomonas sp.B-25 was found to be initiated in the early logarithmic growth phase and reached the maximum when the culture was in the decline phase of growth.

Key words : Psychrotrophic bacteria - Extracellular protease - Proteinase activity.

RESUME

EFFETS DE QUELQUES FACTEURS NUTRITIONNELS ET ENVIRONNEMENTAUX SUR LA PRODUCTION DE PROTÉASE EXTRACELLULAIRE PAR PSEUDOMONAS SP.B-25

Un milieu de base à 1 % Tryptone supplémenté par 0,25 % d'extrait de levure conduit à une production 2,6 fois plus élevée

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de protéase par Pseudomonas sp. B-25. Il n'a pas été possible de remplacer l'extrait de levure-Tryptone dans le milieu par d'autres ingrédients équivalents. Parmi les différents sels inorganiques incorporés dans le milieu de base modifié (1 % de Tryptone + 0,25 % d'extrait de levure) l'addition de 0,2 % de sels de phosphate de potassium provoque une production maximale d'enzyme. Pourtant, aucun des sucres fermentescibles utilisés à titre d'essai comme supplément dans le milieu TYEP (tryptone-yeast extract phosphate) s'est avéré utile. Une production maximale de protéase dans TYEP a été obtenue à pH 7,0 avec inoculation avec 2,5 % de bouillon de culture âgé de 24 h et 48 h d'incubation à 22° C dans un agitateur rotatif. La production de protéase par Pseudomonas sp. B-25 démarre pendant le stade initial de la croissance logarithmique et le maximum de la production a été constaté quand la culture se trouva vers la fin de la phase logarithmique de croissance.

Mots clés : Bactéries psychrotrophes - Protéase extracellulaire - Activité de la protéinase.

I. INTRODUCTION

The widespread adoption of refrigerated storage of bulk raw milk and milk products has largely eliminated spoilage associated with the growth of lactic acid acid bacteria and most pathogens. However, despite these advantages, the storage and handling of milk at refrigeration temperature is selective for psychrotrophic bacteria which have considerable spoilage potential. The psychrotrophs can multiply at or below 7° C, irrespective of their optimum growth temperature, and the increase in total counts observed during storage of raw milk at 7° C is caused by multiplication of its psychrotrophic microflora (Thomas, 1974 ; Cousin *et al.*, 1977).

Protease producing psychrotrophic gram negative bacteria are often isolated from milk (Purschel and Pollack, 1972 ; De Beaukelar *et al.*, 1975) and *Pseudomonas* is the genus most frequently encountered. Several workers (Skean and Overcast, 1960 ; Mc Caskey and Babel, 1966 ; Adams *et al.*, 1975 ; Cousin and Marth, 1977) have isolated protease from psychrotrophic species of *Pseudomonas, Enterobacter, Flavobacterium, Achromobacter, Lactobacillus, Micrococcus and Alcaligenes.*

The object of the present study was to investigate the effects of some nutritional and environmental factors and pattern of protease elaboration by *Pseudomonas* sp.B-25 isolated from a butter sample.

II. MATERIALS AND METHODS

Culture: twenty samples each of raw bulk cooled milk, raw cream, flavoured milk, ice-cream mix, butter and Cheddar cheese were plated with tryptone yeast-extract agar fortified with 10 % reconstituted skim milk. Plates were incubated at 7° C for 10 days and colonies indicating proteolysis were selected and purified by repeated streakings and incubation at 7° C. Thirty-nine of the most proteolytic cultures were examined using the diagnostic tables of Cowan and Steel (1976) and Bergeys Manual of Determinative Bacteriology (1974). An isolate from a butter sample showing highest proteolytic activity and its protease exhibiting comparatively maximum heat resistance (retained more than 36 % of its activity at 70° C for 10 min) and identified and designated as *Pseudomonas* sp. B-25 was used in this study.

Preparation of Inoculum: one hundred milliliters aliquots of the basal medium in 500 ml Erlenmeyer flasks were inoculated with a loopful of the 48 h old slant cultures and incubated on a gyratory shaker for 24 h. The absorbance of the broth culture at 525 nm was adjusted to 0.30 and 2 % inoculum of this culture was used throughout the production studies.

Enzyme Assay: cells were removed from broth cultures by centrifugation at 20,000 × g for 15 min and protease activity of the cell-free broth was determined according to the method of Keay and Wildi (1970) with some modifications. To one ml of the substrate (1 % casein in 0.05M sodium phosphate buffer, pH 7.0), added 1.0 ml of suitably diluted culture supernatant and the reaction mixture was incubated at 37° C for 10 min. The reaction was terminated by adding 2.0 ml of 0.4M trichloroacetic acid (TCA) and the precipitated proteins removed by filtration through Whatman N° 1 filter paper. To one ml of the filtrate obtained after TCA precipitation, 5.0 ml of the sodium carbonate solution (0.4M) was added followed by 1.0 ml of (1N) Folin's reagent and incubated at 37° C for colour development. The intensity of the bue colour developed was measured at 660 nm in Systronics Spectrophotometer 106 (MK.II).

Unit of Activity: The protease activity of the enzyme was expressed in terms of units where a unit is defined as the amount of enzyme required to release TCA soluble fragments giving blue colour equivalent to one µg of tyrosine under the conditions of the assay.

A. Effect of nutritional factors

Nitrogenous Organic Nutrients: various nitrogenous ingredients used as substitutes for tryptone in the basal medium (1 % tryptone + 0.25 % yeast extract) for studying their effect on the enzyme

production were peptone, neo-peptone, proteose-peptone, soytone, and tryptose at 0.5 % and 1.0 % level. Malt extract and beef extract at 0.25 % and 0.50 % level were evaluated as substituents for yeast extract.

Inorganic Salts: NaCl, $MgSo_4.7H_2O$, $(NH_4)_2SO_4$ at 0.25 % and 0.50 % concentrations, $(NH_4)_3PO_4$ and phosphate mixture 1:1 (KH_2PO_4 + $K_2HPO_4.3H_2O$) at 0.1 % and 0.2 % level, and a salt mixture containing 0.01 % $FeSO_4.7H_2O$, 0.1 % NH_4Cl, 0.25 % $MgSO_4.7H_2O$, 0.001 % $MnSO_4.H_2O$ and 0.5 % KH_2PO_4, were added to the basal medium (1.0 % tryptone + 0,25 % yeast extract) to assess the ionic requirements for protease production.

All the above media were sterilized by autoclaving at 15 psi (121° C) for 20 min.

Sugars: glucose, fructose, xylose, mannitol, lactose, sucrose, maltose, arabinose and melibiose at 0.5 % level were added to the tryptone yeast extract phosphate medium to assess their effect on enzyme elaboration. The sugar solutions (5 %) were sterilized separately by filtration through a membrane filter (0.1 μM) and added aseptically to the autoclaved medium before use.

One hundred milliliters aliquots of the different media in 500 ml Erlenmeyer flasks were seeded with 2 % inoculum and incubated at 22° C on a gyratory shaker. The protease activity at 37° C was assayed after 24, 48, and 72 h.

B. Effect of some environmental factors

The influence of different environmental factors like initial pH of the medium, agitation, size of inoculum and temperature and time of incubation was studied in the formulated TYEP medium containing 1.0 % tryptone, 0.25 % yeast extract and 0.2 % potassium phosphate (K_2HPO_4 + KH_2PO_4 1:1).

Initial pH of the Medium: the initial pH of the medium was adjusted to different values ranging from 5.0 to 10.0 and after inoculation, the flasks were incubated on a gyratory shaker at 22° C. The protease activity was determined after 24, 48, and 73 h.

Agitation: three sets of flasks of the TYEP (pH 7.0) medium were inoculated with the test organism at 2 % level. One set was incubated statically, the second was agitated intermittentely on a gyratory shaker for 15 min after every 6 h and the third set was continuously agitated on the gyratory shaker (200 rpm). All the flasks were incubated at 22° C for 72 h and the protease activity was assayed at regular intervals.

Temperature and Period of Incubation: culture flasks with TYEP medium were incubated on a gyratory at 15, 22, 30 and 37° C and the protease activity was determined after 24, 48 and 72 h.

Size of Inoculum: the TYEP medium was seeded with different levels of inoculum (0.5 to 5.0 %) and incubated on a gyratory shaker at 22°C. The enzyme activity was determined at 24 h intervals upto 72 h.

C. Relationship between growth and protease production

One hundred milliliters aliquots of the sterile TYEP medium (pH 7.0) dispensed into 500 ml Erlenmeyer flasks were inoculated at 2.5 % level with a 24 h old culture (absorbance 0.30 at 525 nm) and incubated on a gyratory shaker at 22° C for 72 h. Samples drawn aseptically at regular intervals were assayed for protease activity and viable counts were obtained on tryptone dextrose yeast-extract agar by incubating the plates at 22° C for 48 h.

III. RESULTS

A. Effect of some nutritional factors

Elaboration of the enzyme as a function of yeast-extract concentration is given in Table 1. Though maximum enzyme

TABLE 1 — TABLEAU 1

Effect of concentration of yeast extract on the production of extracellular protease by *Pseudomonas* sp. B-25 in 1 % Tryptone Broth.

Effet de la concentration en extrait de levure dans le milieu à 1 % de tryptone sur la production de protéase extracellulaire par Pseudomonas *sp. B-25*

Concentration of yeast extract (%)	Enzyme activity (units/ml) at different incubation periods (h)		
	24	48	72
0	60	85	65
0.05	160	165	120
0.10	180	180	120
0.15	183	190	130
0.20	189	210	140
0.25	195	218	140
0.30	207	222	142
0.35	207	225	172
0.40	210	225	180
0.45	222	230	180
0.50	222	237	190
0.75	198	210	150
1.00	171	195	125

TABLE 2 — TABLEAU 2

Effect of different nitrogenous nutrients on the production of extracellular protease by *Pseudomonas* sp. B-25

Effet des différents nutriments azotés sur la production de protéase extracellulaire par Pseudomonas *B-25*

Nitrogenous nutrient	Conc. (%)	Enzyme activity (units/ml) at different incubation periods (h)		
		24	48	72
Basal medium*	—	200	225	182
Substitutes for tryptone				
Peptone	0.50	80	95	92
	1.00	110	145	140
Neopeptone	0.50	92	160	107
	1.00	105	30	15
Proteose peptone	0.50	85	90	75
	1.00	106	182	140
Soytone	0.50	56	75	45
	1.00	97	95	80
Tryptose	0.50	115	110	85
	1.00	190	200	175
Substitutes for yeast extract				
Malt extract	0.25	85	175	140
	0.50	5	20	18
Beef extract	0.25	160	160	140
	0.50	115	150	135

* 1.0 % tryptone + 0.25 % yeast extract.

production by the test organism was observed in the medium containing 0.5 % yeast-extract, the enzyme activity at 0.25 % level was not significantly different. Replacement of tryptone in the modified basal medium (1 % tryptone + 0,25 % yeast extract) with peptone, neopeptone, proteose-peptone, soytone or tryptose (0.5 % and 1.0 % level), and of yeast extract with malt extract or beef extract (0.25 % and 0.50 % level) resulted in lowering the protease production (Table 2). Tryptose, however, elicited enzyme secretion similar to that obtained by tryptone.

TABLE 3 — TABLEAU 3

Effect of different inorganic salts on the production of extracellular protease by *Pseudomonas sp.* B-25

Effet des différents sels minéraux sur la production de protéase extracellulaire par Pseudomonas *sp. B-25*

Salt added to the basal medium*	Conc. (%)	Enzyme activity (units/ml) at different incubation periods (h)		
		24	48	72
None	—	202	250	195
$MgSO_4$	0.25	50	170	145
	0.50	5	8	0
$(NH_4)_2SO_4$	0.25	195	250	230
	0.50	177	220	187
NaCl	0.25	192	250	216
	0.50	180	220	210
$CaCl_2$	0.25	255	277	255
	0.50	220	220	172
$(NH_4)_2HPO_4$	0.10	232	255	225
	0.20	245	290	250
K_2HPO_4 + KH_2PO_4 (1:1)	0.10	242	260	240
	0.20	235	292	248
Salt mixture**	0.86	140	245	205

* 1.0 % tryptone + 0.25 % yeast extract.

** Salt mixture consisted of 0.1 % NH_4Cl, 0.01 % $FeSO_4$, 0.25 % $MgSO_4$ 0.001 % $MnSO_4$ and 0.5 % KH_2PO_4.

Among the different inorganic salts incorporated in the modified basal medium (Table 3), addition of $CaCl_2$ at 0.25 % level and $(NH_4)_2HPO_4$ or K_2HPO_4 + KH_2PO_4 (1:1) at 0.20 % concentration enhanced protease production by 13.7 %, 20.2 %, and 23.2 %, respectively.

Supplementation of tryptone-yeast-extract-phosphate (TYEP) medium at pH 7.0 with glucose, fructose, galactose, xylose, arabinose, mannitol, lactose, sucrose, and maltose at 0.5 % level inhibited the enzyme production by Pseudomonas sp. B-25 (Table 4).

TABLE 4 — TABLEAU 4

Effect of sugars on the production of extracellular protease by *Pseudomonas* sp. B-25

Effet de différents sucres sur la production de protéase extracellulaire par Pseudomonas *B-25*

Suggars (0.5 %) added to formulated medium*	Enzyme activity (units/ml) at different incubation periods (h)		
	24	48	72
None	215	285	220
Glucose	77	150	135
Fructose	87	175	110
Galactose	80	170	95
Xylose	130	137	112
Arabinose	117	80	80
Mannitol	117	180	165
Lactose	120	145	110
Sucrose	73	150	115
Maltose	72	175	150

* 1.0 % tryptone + 0.25 % yeast extract + 0.1 % K_2HPO_4 + 0.1 % KH_2PO_4.

B. Effect of environmental factors

Protease production by the test organism was studied in TYEP medium initially adjusted to different hydrogen ion concentrations. Maximum activity (255 units/ml was obtained at pH 7.0 after 48 h incubation at 22° C on a gyratory shaker *(Table 5)*. Very little protease activity was detected in the medium with an initial pH of 10.0. Continuous agitation was found to be obligatory for enzyme elaboration by *Pseudomonas* sp. B-25 since stationary as well as intermittently agitated cultures failed to synthesize any significant level of the enzyme even after 72 h of incubation *(Table 6)*.

Protease production by the test culture increased by 18.5 % after 48 h when the incubation temperature was raised from 15 to 22° C *(Table 7)*. Further increase in incubation temperature hap an adverse effect on enzyme elaboration. The enzyme activity at 37° C after 48 h incubation on a gyratory shaker was only 17 % of that obtained at 22° C for 12 h and subsequent transfer to 15° C for another 36 h or initially at 30° C for 12 h and then at 22° C resulted in decreased enzyme production. Maximum protease activity (320 units/ml) was observed after 48 h incubation on a gyratory shaker at 22° C

TABLE 5 — TABLEAU 5

Effect of initial pH of the TYEP medium* on the production of extracellular protease by *Pseudomonas* sp. B-25

Effet du pH initial du milieu TYEP sur la production de protéase extracellulaire par Pseudomonas *sp. B-25*

pH	Enzyme activity (units/ml) at different incubation periods (h)		
	24	48	72
5.0	92	190	145
5.5	142	195	135
6.0	150	195	145
6.5	178	180	130
7.0	220	255	205
7.5	200	215	185
8.0	190	210	182
8.5	175	180	150
9.0	160	165	150
10.0	7	82	70

* The formulated tryptone yeast extract phosphate medium.

TABLE 6 — TABLEAU 6

Effect of agitation on the production of extracellular protease by *Pseudomonas* sp. B-25

Effet de l'agitation sur la production de protéase extracellulaire par Pseudomonas *sp. B-25*

Incubation	Enzyme activity (units/ml) at different incubation periods (h)		
	24	48	72
With agitation	275	305	255
Intermittent agitation	15	43	35
Without agitation	4	18	30

TABLE 7 — TABLEAU 7

Effect of incubation temperature on the production of extracellular protease by *Pseudomonas* sp. B-25

Effet de la température d'incubation sur la production de protéase extracellulaire par Pseudomonas *sp. B-25*

Temperature (°C)	Enzyme activity (units/ml) at different incubation periods (h)		
	24	48	72
15	74	270	265
22	280	320	255
22* -- 15	158	186	246
30	185	165	122
30** -- 22	215	230	195
37	55	55	15

* Incubated for 12 h at 22° C and subsequently at 15° C.
** Incubated for 12 h at 30° C and subsequently at 22° C.

TABLE 8 — TABLEAU 8

Effect of size of inoculum on the production of extracellular protease by *Pseudomonas* sp. B-25

Effet de la dose d'inoculum sur la production de protéase extracellulaire par Pseudomonas *sp. B-25*

Inoculum size (%)	Enzyme activity (units/ml) at different incubation periods (h)		
	24	48	72
0.5	245	280	230
1.0	250	295	235
1.5	265	300	245
2.0	275	305	255
2.5	275	320	265
3.0	260	300	270
4.0	250	295	252
5.0	235	295	260

Maximum protease activity by the test culture was observed after 48 h incubation at 22° C when the medium was seeded with 2.5 % inaculum of 24 h old culture *(Table 8)*. Seeding the TYEP medium with 3.0, 4.0 and 5.0 % inoculum showed slight decrease in protease activity.

C. Relationship between growth and enzyme production

The protease activity was initiated in the early logarithmic phase and reached maximum level after 48 h incubation when the culture was in decline phase of growth (fig. 1). Subsequently enzyme production declined upto 60 h before reaching a plateau.

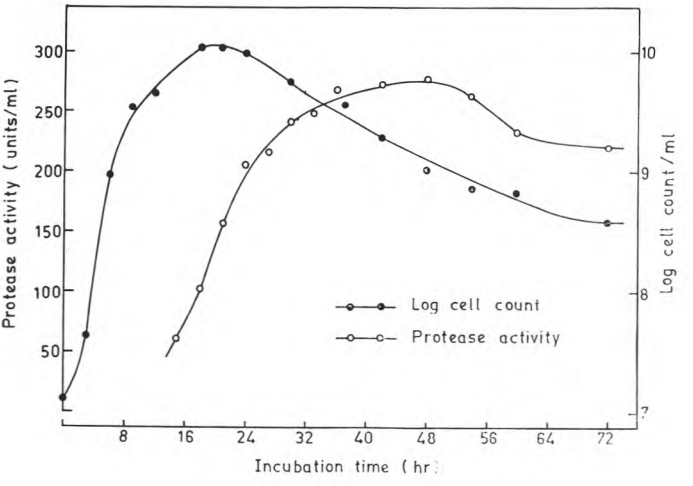

fig. 1

Correlation between growth and protease production by *Pseudomonas sp.* B-25.

The TYEP medium (pH 7.0) was inoculated at 2.5 % level with 24 h old broth culture (absorbance 0.30) and incubated at 22° C on a gyratory shaker.

Relation entre la croissance cellulaire et la production de protéase par Pseudomonas *sp. B-25*

IV. DISCUSSION

Protease production *by Pseudomonas* sp. B-25 was initially studied in a basal medium containing 1 % tryptone when incubated on a gyratory shaker at 22° C. Substitution of various nitrogenous

media ingredients such as peptone, neopeptone, proteose-peptone, soytone and tryptose for tryptone failed to increase the enzyme production in the basal medium. Nigam et al. (1981) also reported maximum production of protease by *Pseudomonas aeruginosa* in the medium containing tryptone. Supplementation of the 1 % basal medium with 0.25 % yeast extract increased the enzyme production by 2.6-fold. However, substitution of yeast extract with malt extract or beef extract resulted in decreased enzyme production. Stimulation in enzyme production in the presence of yeast extract has also been observed in *Bacillus stearothermophilus* 1503 (O'Brien and Campbell, 1957) and *B. subtilis* (Boyer and Carlton, 1968). The increase in cell growth and enzyme production by the incorporation of yeast extract in the medium is probably due to the fact that it supplies a variety of organic growth factors required by microorganisms. The results of the present study are, however, at variance with the findings of Millet et al. (1969) who reported complete repression of enzyme biosynthesis in *B. megaterium* when yeast extract was incorporated in the minimal medium.

Whereas Juffs (1976) obtained maximum proteinase production by two *Pseudomonas* spp. in a peptone basal medium, inhibitory effect of peptone on enzyme synthesis when added at a concentration exceeding 0.5 % has been reported in *B. mycoides* and *B. subtilis* by Pavlasova and Starka (1959). Proteinase synthesis in two *Pseudomonas* spp. was decreased in media containing either sodium caseinate, Hammersten casein or lactalbumin as the sole organic constituents (Juffs, 1976). In contrast, Rowe and Gilmour (1983) observed a cooperative stimulatory effect of whey and isoelectric casein on extracellular protease production by *Ps. fluorescens* B-52.

When the tryptone yeast extract medium was supplemented with different inorganic salts individually, 16 to 20 % stimulation in extracellular protease production by *Pseudomonas* sp. B-25 was noted with K_2HPO_4 and KH_2PO_4 (1:1) at 0.20 % level. Other salts including assorted salt mixture were either inhibitory or had no effect on enzyme production by the test organism. Though McDonald (1961) reported a stimulation of protease production by a *Micrococcus* sp. ATCC N° 407 in the presence of NaCl, no such stimulation in enzyme production was observed in the present study. Nigam et al (1981), in fact, observed an inhibitory effect of sodium chloride on protease production by *Pseudomonas aeruginosa.*

All the sugars assessed as supplements to the formulated TYEP medium exerted inhibitory effect on protease production by *Pseudomonas* sp B-25. Repression of proteinase synthesis by glucose has been reported in *Serratia* sp. (Ryden and Hofsten, 1968), *Aeromonas proteolytica* (Litchfield and Prescott, 1970), *Pseudomonas fluorescens* (Juffs, 1976) and *Ps. fluorescens* 32A (Mc Kellar, 1982).

Pseudomonas sp. B-25 elaborated maximum extracellular protease when the initial pH of the TYEP medium was adjusted to 7.0. The enzyme level gradually declined at progressively higher values. Juffs (1976) had also obtained maximum proteinase production in the case of *Pseudomonas fluorescens* and Ps. aeruginosa in peptone yeast extract broth at pH 7.0 ± 0.2.

Production of extracellular protease by *Pseudomonas* sp. B-25 was maximum when the organism was grown at 22° C for 48 h. While studying the effect of temperature on proteinase production by two *Pseudomonas* spp., Juffs (1976) reported that *Ps. fluorescens* proteinase production declined progressively as the incubation temperature was reduced from 20 to 5° C, whereas protease production by *Ps. aeruginosa* was maximal at 30° C. A shift down in incubation temperature from 22 to 15° C or 30 to 22° C after 12 h and subsequent incubation for another 36 h failed to enhance the protease production by *Pseudomonas* sp. B-25. This is in contrast to the findings of Sastry and Mathur (1979) who showed that maximum milk clotting enzyme was produced by *B. megaterium* K-40 when the culture was initially incubated at 37° C for 12 h and subsequently at 30° C for 24 h.

Since Pseudomonas are obligate aerobes, continuous aeration is generally required for their optimal growth and enzyme production. *Pseudomonas* sp. B-25 also exhibited maximum protease production when incubated at 22° C for 48 h on a gyratory shaker. Similar observations were made in the case of a marine bacterium *Aeromonas proteolytica* N by Merkel et al. (1964). The low enzyme production under some of these conditions might simply reflect a failure of the bacteria to grow under the experimental conditions.

The elaboration of an enzyme exhibits a characteristic relationship with regard to the growth phase of the enzyme producing organism. The production of protease by *Pseudomonas* sp. B-25 was observed to be initiated in the logarithmic growth phase and reached the maximum when the culture was in decline phase of growth. In so far as proteases are concerned, Keay et al. (1972) showed that production of neutral proteases by sporeforming bacilli reached their highest activity before maximal cell count was attained, whereas maximal production of alkaline proteases was achieved after the stationary growth phase. Driessen (1981) reported that it is only towards the end of the logarithmic growth phase of *Ps. fluorescens* 22F does proteinase accumulate in the milk. Similarly, Mc Kellar (1982) observed that the maximum rate of extracellular proteinase production by *Ps. fluorescens* strain 32A occurred during the late log and early stationary phases of growth at both 55 and 20° C.

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