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Determination of Proteolytic activity of Psychrotrophic *Pseudomonas* species isolated from dairies of Aurangabad on pH and temperature gradient.

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Abstract

Psychrotrophic bacteria are able to grow at 7^0C or lower but had higher growth temperature called psychrotrophs. They are commonly found in different cold environments such as deep oceans, Antarctic regions, seasonally cold environments as their potential ability to sustain high temperature. Psychrotrophs produce cold active enzymes which reveals higher catalytic activities as compared to mesic and thermic conditions.Most psychrotrophs produce hydrolytic heat stable extracellular enzymes like proteases, lipases, phospholipases etc in milk or dairy products stored at low temperature. In this present study cooled buffalo milk was used for the isolation of psychrotrophic *pseudomonas* species and its protease production was studied on different pH and temperatures. On skim milk agar a zone of hydrolysis showed proteolytic activity of *Pseudomonas* and it is measured by selection ratio.Selection ratio(SR) = diameter of zone of hydrolysis / diameter of colony in mm. proteolytic activity was optimized on different pH and temperatures. Results showed that maximum protease production was observed at 37^0C and pH 7.0 which is 6.0 ± 0.1 SR value. Psychrotrophic bacteria was also able to produce protease at refrigeration temperature and prolonged cooling can alter the quality of milk.

Keywords: Psychrotroph, protease, selection ratio, zone of hydrolysis.

Introduction

Psychrotrophic bacteria are those which grow at 7^0C or lower but had higher growth temperature called psychrotrophs. They are commonly found in different cold environments such as deep oceans, arctic regions, seasonally cold environment as their potential ability to sustain high temperature. Psychrotrophs produce cold active enzymes which reveals higher catalytic activities as compare to mesic and thermic conditions. Now a days psychrotrophs have been widely used

for their cold active enzymes in biotechnological industries. Most psychrotrophs produce hydrolytic heat stable extracellular enzymes like proteases, lipases, phospholipases etc in milk or dairy products stored at low temperature. Though pasteurization kill most of the psychrotrophic bacteria but do not degrade their extracellular metabolites. These metabolized subsequently degrade the quality of milk. The hydrolytic extracellular enzymes degrade the important milk constituents and causes spoilage of milk and milk products. Different psychrotrophic bacteria have been isolated from milk and milk products which includes *Acinitobacter*, *Achromobacter*, *Burkholderia*, *Stenotrophomonas*, *flavobacterium*, *Psedomonas*, *Micrococcus*, *Alcaligens* etc. In prolonged refrigeration of milk and milk products psychrotrophic bacteria become dominant. Most studied psychrotrophic bacteria in milk and milk products is Pseudomonas. Different hydrolytic enzymes produced by Pseudomonas have been reported. Pseudomonas is a proteolytic organism involved in low temperature spoilage of dairy foods. Protease are known for use in various industries such as leather, food and detergent etc. Psychrotrophic microorganisms secrete the enzyme with some distinct feature such as high activity at low temperature and rapid degradation etc. These properties made the enzyme ideal for use in detergent industries, leather processing,food industries, and biotechnological industries. Production of protease greatly affected by media composition such as temperature, inoculum size and pH. In industries to reach maximum yield and to reduce the production cost, optimization is necessary. Use of locally isolated bacteria in various industries can reduce the energy consumption and utilization cost. Several studies were carried out on protease production using different microorganisms like *Pseudomonas*, *Bacillus subtilis*, *Curatobacterium*, *Pedobacter* etc. Cost cutting in enzyme production is still unsolved problem. In this paper we attempt to study the effect of temperature on protease production by psychrotrophic Pseudomonas isolated from refrigerated milk of local dairy in Aurangabad, Maharashtra, India.

Material and Methods

Isolation of Psychrotrophic *Pseudomonas.*

Sample of cooled buffalo milk was obtained from different dairies of Aurangabad and collected in sterile screw cap tube and kept in refrigerator. Collected samples were assigned distinct codes.Serial dilutions of samples were prepared (10^{-1} to 10^{-10}) in sterile distill water and plated on nutrient agar. On incubation of plates at 7^0C temperature for 10 days isolated colonies were picked up for colony characters and biochemical tests. Identification of *Pseudomonas* was done on the basis of colony characters and biochemical characters using Bergey's manual of systematic bacteriology.

Isolated *Pseudomonas* was screened for protease production. 0.1 ml of active culture of *Pseudomonas* was spread on sterile skim milk agar and incubated for 10 days at 7^0C. A zone of clearance around the colonies shows proteolysis. Diameter of the colonies and zone of hydrolysis surrounding the colonies were measured and selection ratio was determined by the formula

Selection ratio(SR) = diameter of zone of hydrolysis in mm / diameter of colony in mm

Furthercolonies were studied for protease production by using the selection ratio formula for the parameters like temperature and pH.

Effect of temperature and pH on protease production

Active culture of proteolytic isolates were streaked on sterile skim milk agar plates at temperature of 7^0C,15^0C, 24^0C, 37^0C and 55^0C and different pH of 5, 6, 7, 8, 9, 10 for 24hours. After incubation selection ratio (S:R) was calculated for each plate by using above formula.

Result and discussions

Sample was collected and coded appropriately (BMS101) for further study. Isolated colonies were assigned the numbers and five colonies were selected for study

Table 1: Colony characters of the isolates

colony	Size in mm	Shape	Color	Margin	Opacity	Elevation	consistency

P1	1.0	Circular	White	Entire	Opaque	Convex	Mucoid
P2	1.0	Circular	Off white	Entire	Opaque	Convex	Mucoid
P3	2.0	Circular	White	Entire	Opaque	Flat	Mucoid
P4	1.0	Circular	Golden yellow	Entire	Opaque	Convex	Mucoid
P5	1.0	Circular	Colorless	Entire	Semi translucent	Flat	Mucoid

Table 2: Grams nature, morphology and spore production by the isolates

Colony	Grams nature	Morphology	Spore production
P1	1.0	Circular	White
P2	1.0	Circular	Off white
P3	2.0	Circular	White
P4	1.0	Circular	Golden yellow
P5	1.0	Circular	Colorless

On the basis of colony characters colony P1, P2, P4 are selected for biochemical characteristics. All the selected colonies ferment different sugars and other chemicals as shown in table 3 below.

Colony	Glucose	Inulin	Lactose	Maltose	Mannitol	Indole	MR	VP	Citrate	Urease	Nitrate reduction
P1	-	-	-	-	+	-	-	-	+	-	+
P2	-	+	-	-	-	+	-	+	-	+	-
P3	+	-	-	-	+	-	+	-	-	-	+
P4	-	-	-	-	+	+	-	-	+	-	+

P5	-	+		+	-	-	-	-	-	+	-

On the basis of biochemical characters colony P1 is identified as *Pseudomonas* species by referring Bergey's manual of systematic bacteriology and used for protease production at different temperature and pH.

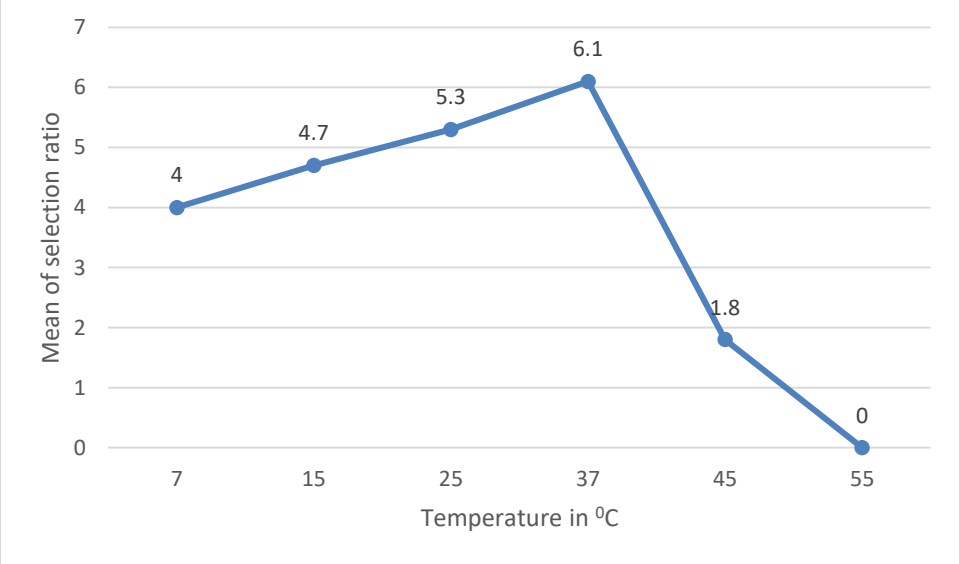
The selection ratio (SR) of protease production by *Pseudomonas* was shown in a table 4 and graph 1 below which clearly indicate that *Pseudomonas*produce protease on pH 7.0 at temperatures 7^0C the selection ratio was 4.0, at 25^0C selection ratio (SR) was 5.3 and at 37^0C was 6.1 respectively. At 45^0C selection ratio (SR) declined to 1.8 and there was no protease production at 55^0C as zone of hydrolysis was not observed.

Table 4: Showing SR (selection ratio) of Psychrotrophic Pseudomonas species at different temperature.

pH	Temperature	Selection Ratio (SR)	Mean of SR
7.0	7^0C	3.8	4.0
		4.2	
		4.1	
	15^0C	4.3	4.7
		4.8	
		5.1	
	25^0C	5.4	5.3
		5.6	
		5.0	
	37^0C	5.9	6.1
		6.3	
		6.1	
		2.1	

	45^0C	1.8	1.8
		1.5	
	55^0C	---	---

Graph 1: Selection ratio of protease production at different temperature on pH 7.0



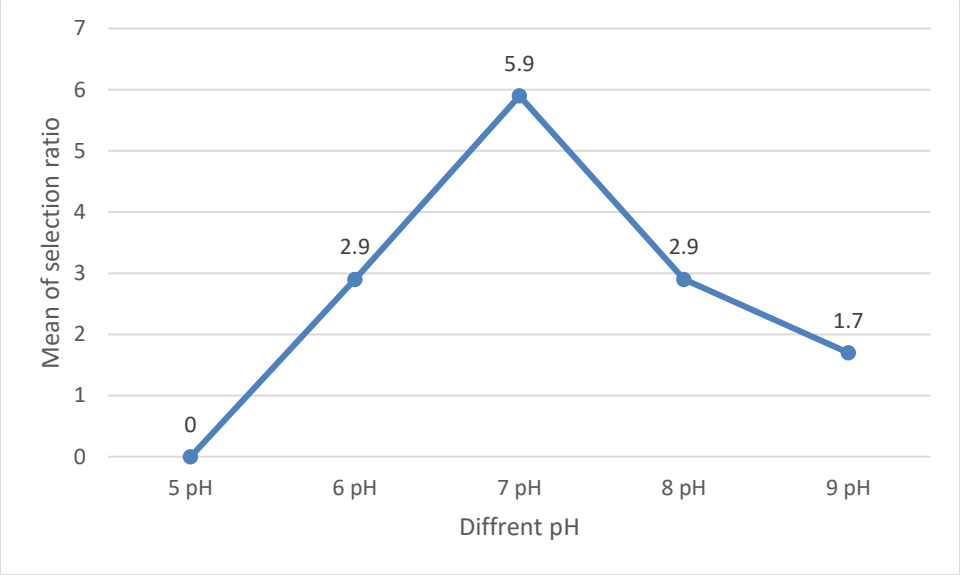
Similarly selection ratio of protease production at 37^0C temperature and different pH was shown in table 5 and graph 2 below. At pH 5.0 protease production was not observed because colonies did not show the zone of hydrolysis. Selection ratio (SR) at pH 6.0 was 2.9, at pH 7.0 maximum SR value was obtained which was 5.9. At pH 8.0 and 9.0 selection ratio were 2.8 and 1.7 respectively.

Table 5: Showing selection ratio (SR) of protease production at different pH on 37^0C temperature.

Temperature	pH	Selection Ratio (SR)	Mean of SR
37^0C	5.0	---	---

	6.0	2.8	2.9
		3.1	
		2.9	
	7.0	6.1	5.9
		5.8	
		5.9	
	8.0	2.7	2.9
		3.2	
		3.0	
	9.0	1.4	1.7
		1.9	
		1.8	

Graph 2: Selection ratio (SR) of protease production at different pH on temperature 37^0C.



Conclusion

Tables and graphs clearly indicate that isolated *Pseudomonas* produce SR ratio at different temperatures and pH values and maximum selection ratio (SR) at 37^0C and pH 7.0 was observed ie. 6.0 ± 0.1, but the protease production at lower temperature is also observed. Prolonged storage of milk in freezer can alter the milk quality due to secretion of various hydrolytic enzymes.

Different psychrotropic *Pseudomonas* species are able to produce extracellular protease in varying heat stability, pH optima, molecular weight and other properties. Temperature is an important parameter considered for protease production in dairy industries. Protease production is a heat sensitive process because it get inactivated at 55^0C temperature.

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