Protease and Lipase production of psychrotrophic bacteria of dairy origin

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SUMMARY

Psychrotrophic bacteria were isolated from raw milk samples by incubation at 5°C — 7°C. Viable counts of these bacteria were carried out and visible colony forming units were found after 7 days and 10 days at 5°C — 7°C. Isolates were tested for production of the extra-cellular enzyme lipase and protease. In each case enzyme positive cultures were purified and tested to identify to a generic level. The optimum temperature for enzyme production was tested for and was generally 30°C. The temperature at which the organisms were grown had effect on enzyme production.

INTRODUCTION

A trend was observed in storage of raw milk at refrigeration temperature for two days or longer on the farm and at the processing plants prior to heat treatment. Psychrotrophs become the predominant micro flora of the raw milk. Psychrotrophs can be defined as the microorganism having the ability to grow rapidly at refrigeration temperature (3°C-7°C) irrespective of the known optimum temperature ranging between 20°C — 30°C (Marshall, 1979). Most of psychrotrophs bacteria are gram negative rods, non-spore formers of the genera, Pseudomonas, Flavobacterium, Alcaligenes, Entrobacteriaceae and Chromo-bacterium spp (Murray and Steward, 1978; Muir et al., 1979). Sporing and non-sporing gram-positive organisms both have also been isolated and these were Bacillus, clostridium, Micrococcus, Corynebacterium, Streptococcus and Arthrobacter spp (Collins, 1981; Griffiths et al., 1981; Johnson and Bruce, 1982).

Most of psychrotrophs bacteria are destroyed by pasteurization (except spore formers), never-the-less they produce extra cellular enzymes, which are extremely, heat stable (Sorhaug and Stepaniak, 1991, Champagne et al.,

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1994; Zahran and Ward, 2001). Thus, the microbial enzymes, which survive
the heat treatment, can affect the quality of many heat-treated dairy products
by causing deterioration of flavor during the storage of the products
(Fitzratrick, 2001).
The objectives of this study were to isolate proteolytic and lipolytic
bacteria of dairy origin and to study their enzymes activity.

MATERIALS AND METHODS

**Incubation of the milk sample:**
A raw milk sample was incubated in a cold room with temperature
ranging from 5°C to 7°C for seven days then the following tests were carried
out.

**Estimation of viable count:** proteolytic and Lipolytic counts using the
surface plate method as reported by Luck (1972). A total viable count for all
colonies and counts of proteolytic or lipolytic colonies were carried out on
skimmed milk agar and Victoria blue margarine agar respectively.

**Identification of the isolates:** proteolytic and lipolytic isolates were
classified according to Bergey's manual of determinative bacteriology
(Buchman and Gibbson, 1974) and the Gram negative rod-shaped bacteria
were identified using the first stage diagnostic procedure of Cowan and
Steel (1965).

**Enzyme production:**
The method described by Griffiths et al., (1981) was used for
production of protease and lipase enzymes. Organisms were grown in
Skerman's mineral salts medium at 30°C, 25°C, and 4°C for fourteen days.
The supernatants from these cultures were used as a source of Protease and
Lipase enzymes.

**Enzyme Assay:**
Lawrence et al., (1967) agar diffusion method was adopted with
slight modifications. The activity of the enzymes was tested in 30% skim
milk agar as a substrate for protease and tributyrin agar for Lipase. The
substrate medium was poured as a thin layer for protease and over nutrient
agar for lipase. Holes 4mm in diameter were cut in the agar with a sterile
cork borer. 2011l of enzyme sample were placed
in the holes using a micro syringe. Five holes were used per isolate /
temperature (the diameter of the zone is an average of five holes). The plates
were covered and incubated at 30°C for 7 days. Enzyme activity appeared as
a clear zone or a precipitation around the holes. The diameters of the zones
were measured.
RESULTS AND DISCUSSION

Total viable counts and proteolytic and Lipolytic counts were recorded after ten days incubation at 5°C — 7°C. (Table1). Percentage of the total viable count to proteolytic count, lie between 14% — 15% and total viable count to lipolytic count between 10% — 11% almost similar. However, it appears from the result obtained, psychrotrophic counts of more than 106 cfu /ml in raw milk can result in production of these enzymes. Matselies and Rouissis (1998) observed maximum protease and lipase activities when bacterial count were 108 — 109 and 107 — 108 cfu /ml respectively. Psychrotrophic microorganisms isolated from raw milk were Alcaligenes, pseudomonas, Bacillus spp. and Entrobacteriaceae. Pseudomonas spp. was found to be the predominant species synthesizing these enzymes. From the four organisms isolated Bacillus spp. showed the highest protease activity of all four organisms. The highest activity was observed when the organism was grown at 30°C and enzyme tested at the same degree of temperature. When the organism was grown at 30°C and the enzyme was tested at 25°C, the drop in activity of protease was only 0.6%. A high drop in activity (42.7%) was observed when the organism was grown at 30°C and enzyme tested at 4°C. so the temperature at which the enzyme was tested appeared to have a bearing on the enzyme activity. Adams et al., (1975) observed that optimum temperature for protease activity of different psychrotrophic bacteria lay between (40°-45°C). This explains why protease of four isolates tested showed a high activity at 30°C. From these results (Table 2 and 3) it can be assumed that optimum enzyme activity is related to optimum growth temperature. It can also be assumed that the Bacillus spp. is a high mesophilic psychrotrophs, although growing and producing protease at 4°C would appear to grow better and produce more protease at 25°C with the best at 30°C. From this assumption the Entrobacteriaceae could be termed a mid, mesophilic psychrotrophs, because its best activity was at the 25°C level. Similarly the Pseudomonas spp. (organism 2) could be considered a psychrophilic psychrotrophs since its best activity was observed at 4°C.
Table 1. Proteolytic, lipolytic and total viable count (in 30% skim milk and Victoria blue Margarine agar) of psychrotrophic bacteria incubated at 5°C-7°C for 7 and 10 days.

<table>
<thead>
<tr>
<th>Test</th>
<th>Incubation time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>log_{10}cfu/ml</td>
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<tr>
<td>Total viable count/ml (30% skim milk)</td>
<td>7.150</td>
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<tr>
<td>Proteolytic count/ml</td>
<td>NC</td>
</tr>
<tr>
<td>Total viable count/ml (Victoria blue margarine agar)</td>
<td>NC</td>
</tr>
<tr>
<td>Lipolytic count/ml</td>
<td>NC</td>
</tr>
</tbody>
</table>

NC: Not counted (>300)

Table 2. Protease activity of different psychrotrophic bacteria.

<table>
<thead>
<tr>
<th>Temperature at which organisms incubated and lipase produced (°C)</th>
<th>Temperature at which lipase was tested (°C)</th>
<th>Pseudomonas sPP (°C)</th>
<th>Pseudomonas sPP (°C)</th>
<th>Bacillus spp</th>
<th>Entrobacteriaceae</th>
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<tbody>
<tr>
<td>30</td>
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<td>25</td>
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<td>30.33</td>
<td>10.50</td>
<td>39.00</td>
<td>21.00</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>7.95</td>
<td>5.50</td>
<td>22.50</td>
<td>9.60</td>
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<td>17.25</td>
<td>8.30</td>
<td>36.00</td>
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<td>23.00</td>
<td>15.50</td>
<td>10.00</td>
<td>9.50</td>
</tr>
</tbody>
</table>

* Number shown is an average of 10 readings.

For lipase activity *Pseudomonas* spp (organism 3) would appear to be the least active lipase producer of the four isolates but in terms of activity the results showed little variation over the three temperatures at which the isolates were grown (Table 3). Using the same assumption of the connection between enzyme and optimum growth temperature the *Pseudomonas* spp. (organism 4) would appear to be a mesophilic psychrotrophs, as like the *Alcaligenes* spp. The *Entrobacteriaceae* (organism 2) tends to be a psychrophilic Psychrotroph, while the other *Pseudomonas* spp (organism 3)
did not fit into this pattern. From Table (3) lipase activity was better at 25°C and 30°C than at 4°C. Those findings were opposite to the observation of Griffiths (1989) who reported that a maximum lipase activity at 5°C was similar to that at 25°C.

As conclusions, the effective control of Psychrotrophs must begin on the farm and be followed through all the way till the retail stores. Clean equipment and packages, limited time of storage and low holding temperature for raw milk will lower the growth of Psychrotrophs.

**Table 3.** Lipase activity of different psychrotrophic bacteria.

<table>
<thead>
<tr>
<th>Temperature at which organisms incubated and lipase produced (°C)</th>
<th>Temperature at which lipase was tested (°C)</th>
<th>Alcaligenes spp</th>
<th>Pseudomonas spp (3)</th>
<th>Enterobacteriaceae (2)</th>
<th>Pseudomonas spp (4)</th>
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*Number shown is an average of 10 readings*

**ACKNOWLEDGEMENTS**

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REFERENCES


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