Screening of psychrophilic and psychrotropic microorganisms and their β-galactosidase activity at the sub-zero degree temperature

Kazuo KIMURA, Hideo TOHGOH and Hideo KUSAOKE

Screening of psychrophilic and psychrotrophic microorganisms was conducted with using dairy products as the source of selection. Twenty seven kinds of microorganisms were found and tentatively classified into seven groups. Six of them are bacteria and one is a yeast. One of the bacteria designated as P 15 showed the highest intracellular β-galactosidase activity over the wide range of temperature from minus 7.8°C to plus 60°C. However,the activity at the sub-zero degree temperature needs further confirmation.

Introduction

Reports of psychrophilic and psychrotrophic microorganisms have been no more than scanty probably because their growth rates were so slow not as to be suitable for scientific study. More than two decades ago, Ogata[1] inspired the importance of the study of such microorganisms and with the recent advocacy of marine biotechnology,[2] more interests are gradually being focused on the microorganisms that grow at the low temperature as well as on the enzymes that work under the similar condition.[3,4,5]

The present study was undertaken to survey such microorganisms and clarify the properties of their enzymes, specifically in relation to the low reaction temperature.

Materials and Methods

Sources for the selection of microorganisms Commercial milk and processed milk on the market were used as the source for the selection of microorganisms. Other sources used were raw and pasteurized milk obtained from the near-by manufacturer (Nihon Rakunoh, Fukui plant).

Growth medium for the selection of microorganisms The growth media composed of 2% lactose, 0.05% yeast extract (Difco), 0.05% peptone (Difco), 0.02% NaCl and 2% agar with pH values of 7 and 8 were used for the selection of microorganisms.

Incubation condition for the selection of microorganisms The agar plates containing the media described above were placed in a cool incubator with the temperature of plus 5°C over the period of more than 20 days.

Tentative classification of the microorganisms The microorganisms isolated from the colonies

which appeared on the agar plates were microscopically classified with respect to the cell form and the cell size. Pigmentation colors were identified. Gram staining was conducted according to Hucker method.[6] Reference strains were Micrococcus luteus JCM 1464 and Escherichia coli IFO 3301. Temperatures for the survival of the microorganisms were recorded from the repeated culture of their agar slants in the incubators with the temperatures indicated.

Culture condition for cell harvest The liquid medium (designated as L) composed of 2% lactose, 0.2% beef extract (Difco), 0.1% peptone (Nissui) and 0.025% NaCl with the pH of 6.99 was used for the cell harvest. Three hundred ml of the medium were dispensed in a flask of the volume of 500 ml. The sub-culture was conducted in a 30 ml medium of the similar composition in a 300 ml flask except for the omission of lactose, with rotary shaking of 130 rpm for 6 days at 5°C. It was transferred into the harvest medium and was cultured at the similar condition for 5 days after which the cells were harvested with centrifugation and resuspended in 5 ml of 0.1 M phosphate buffer of pH 6.85. The similar sub- and harvest cultures were conducted at 30°C but the culture times were 2 days and 4 days, respectively.

Measurement of β-galactosidase activity The reaction mixture for the measurement of β-galactosidase activity was composed of 0.5 ml of cell suspension in 0.1 M phosphate buffer of pH 6.85 and 1.5 ml of 13.3 mM 2-nitrophenyl-β-D-galactopyranoside (abbreviated as ONPG) in 0.1 M phosphate buffer of pH 6.85 with a total volume of 2.0 ml. The reaction was started with the addition of the cell suspension into the ONPG solution preincubated for 15 min at the temperature designed for the test. After 10 min. of the reaction, 1.0 ml of 0.2 M Na_2CO_3 was added to stop the reaction. After about 30 to 60 min standing at the room temperature of about 30°C, the mixture was centrifuged to remove off the cells and the optical density at 420 nm of the supernatant of the reaction mixture was measured. The enzymic activity is expressed according to the following equation in which 0.00014 is micro-molar

$$\text{iu/ml} = \frac{OD_{420}}{0.00014 \times 10\ (\text{min})} \times \frac{1}{\text{Cell suspension (ml)}}$$

absorption coefficient of o-nitrophenol at 420 nm previously determined experimentally. The cell suspension used is as 60 times concentrated as the original culture broth as is shown in the procedure of cell harvest. In one case, the enzymic activity is expressed per unit weight of the dry cells after drying the cells at 110°C.

Enzymic reaction below 0°C The enzymic reaction at 0°C and sub-zero degree temperature was carried out in a cool water bath containing a non-freezing liquid Nybrine Z_1 (Nissoh

Maruzen chemical) in the volume ratio of 40% to water with a freezing temperature of minus 15.0°C. In order to start the reaction sharply at the designed temperature, a small cup was manually made of aluminum film and inserted into a test tube containing the ONPG solution. The cell suspension was carefully poured into the cup and after preincubating for 15 min at the temperature designed the cell suspension was mixed with the ONPG solution either by breaking the cup by sticking with a needle or by shaking the test tube vigorously. The reaction mixture was composed in two ways, one without glycerol and the other with glycerol. The former was the same as described before but the latter was composed of the ONPG solution containing 20% (V/V) glycerol and the cell suspension.

Enzymic reaction mixture of various pH values For the assay of pH profile of the enzyme, acetic acid and sodium acetate buffer (0.25 M) for the pH range between 3.5 and 5.5, potassium dihydrogen phosphate and disodium hydrogen phosphate buffer (0.25 M) for the pH range between 6.0 and 8.0 and tris-hydroxymethylaminomethane and hydrochloric acid buffer (0.25 M) for the pH range between 8.5 and 9.0 were used respectively. The reaction mixture was composed of 0.5 ml of cell suspension in water and 1.0 ml of 0.25 M respective buffer with a total volume of 2.5 ml. The reaction was started by the addition of the cell suspension and carried on for 10 min at 30°C. Then, 0.5 ml of 0.4 M Na_2CO_3 was added to stop the reaction.

Growth medium for the induction test In order to survey the effect of an inducer for β-galactosidase formation, a liquid medium (designated as S) of the almost similar composition as that for the cell harvest described above except that lactose was omitted was used, namely, of the composition of 0.3% beef extract, 0.1% peptone and 0.025% NaCl with pH 6.98.

Results

Screening of psychrophilic and psychrotrophic microorganisms and their tentative classification During the cool incubation at 5°C a variety of colonies of microorganisms appeared on the agar plates. On the plates streaked with raw milk various fungi developed abundantly making it difficult to isolate specific colonies. Consequently, most of microorganisms were picked up from the plates contacted with pasteurized milk. About twenty seven kinds of microorganisms were isolated and they were tentatively classified into seven types as shown in Table 1. One strain designated as P 14 is unequivocally a yeast. All other six strains are bacteria and Gram staining so far conducted with P 1, P 3, P 15, P 22,and P 25 showed that they were all Gram negative. According to the proposal by Morita[7] in relation to the growth temperature, the strain P 1 is psychrophilic and other five strains P 3, P 14,

Table 1. Types of isolated microorganisms

Isolated Type	Number	Cell Form	Cell Size(μ)	Pigment Color	Survival Temperature 5°	10°	30°	35°
P1	6	rod	0.5 x 3	yellow	+	+	-	-
P3	2	short rod	1 x 3	none	+	+	+	
P14	1	oval	8 x 10	orange	+	+	+	
P15	1	short rod	1 x 3	none	+	+	+	-
P16	3	cocci	1 x 1	none	+			
P22	12	short rod	1 x 2	orange	+	+	+	
P25	2	short rod	1 x 2	yellow	+	+	+	

P 15, P 22 and P 25 should be designated as psychrotrophic. P 16 is not successful to keep living by careless maintenance. The culture broth of these microorganisms showed no more than undetectable activity of β - galactosidase. On the other hand, all showed intracellular activity of β - galactosidase and four strains among them P 1, P 3, P 15 and P 22 were strongest. Hereafter the strain P 15 was used throughout the present study.

Intracellular β - galactosidase activity of the cells grown at 5°C and its temperature profile In most of the liquid culture the cell growth reached around the range between 0.1 and 0.3 mg dry weight per ml of the broth. The cell concentration in most of the enzyme reaction mixture was in the range between 0.5 and 3 mg dry weight per ml of the mixture.

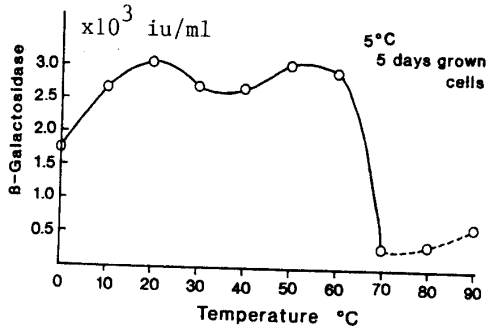

Fig.1. Temperature profile of β - galactosidase activity of the cells grown at 5°C

As shown in Fig.1, the cells grown at 5°C have a strong activity of β - galactosidase over the wide range of temperature between 0°C and 60°C and lose it rapidly around and above 70°C. A small but appreciable amount of activity observed above 80°C must be due to non-enzymic decomposition of the substrate as proved by the parallel experiment without the cells.

Screening of psychrophilic and psychrotropic microorganisms and their β-galactosidase activity at the sub-zero degree temperature

Intracellular β - galactosidase activity of the cells grown at 30°C and its temperature profile Almost unexpectedly the cells grown at 30°C show a similar temperature pattern of β - galactosidase activity. However, the activity at 60°C is remarkably deteriorated compared with the cells grown at 5°C (Fig. 2).

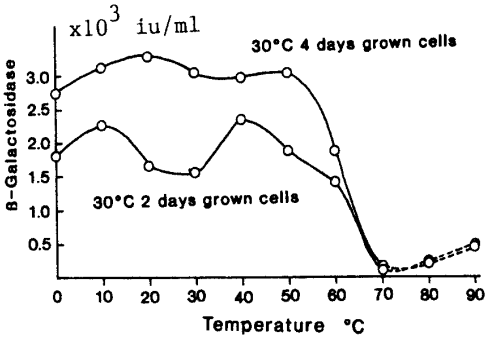

Fig.2. Temperature profile of β - galactosidase activity of the cells grown at 30°C

Intracellular β - galactosidase activity at the sub-zero degree temperature of the cells grown at 30°C The results of the previously described experiments inspired the question if the cells could show β - galactosidase activity at the sub-zero degree temperature. In order to answer this question the following two points must be satisfied, namely

(1) How to adjust the temperature accurately at the time of mixing the reaction solution. and (2) Is it adequate to use Na_2CO_3 for stopping the reaction punctually?

The first problem was met by the devised procedure described in the part of Method. The second problem could be partially solved by avoiding freezing of the reaction mixture with the addition of glycerol.

The apparent enzymic activity observed without the addition of glycerol (Fig. 3 right hand side) is highly problematic whether it really indicates an intrinsic activity or

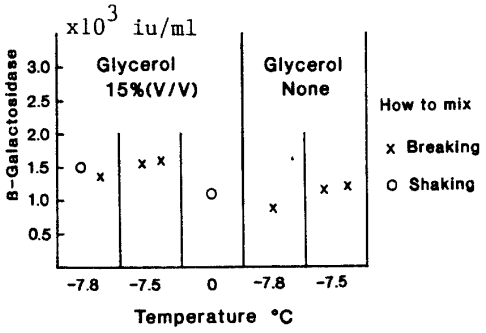

Fig.3. β - galactosidase activity with and without glycerol at the sub-zero degree temperatures of the cells grown at 30°C

only pseudo-activity during the process of defreezing of the reaction mixture.

pH profile of β - galactosidase activity and the constitutive nature of the enzyme formation With using the two kinds of cells grown at 30°C either in the medium containing lactose (L Medium) or in the medium without lactose (S Medium), β - galactosidase activity was examined in the range of pH 3.5 to pH 9.0. Both kinds of the cells showed the optimal activity between pH 6.5 and 7.5 (Fig. 4). The cells grown in the medium not containing lactose showed higher β - galactosidase activity than those grown with lactose. It might be due to the pH of the broths at the time of harvest. The pH of the former was 8.6 and that of the latter was 4.2. The results obtained proves clearly that the formation of β - galactosidase is constitutive in this strain of bacteria.

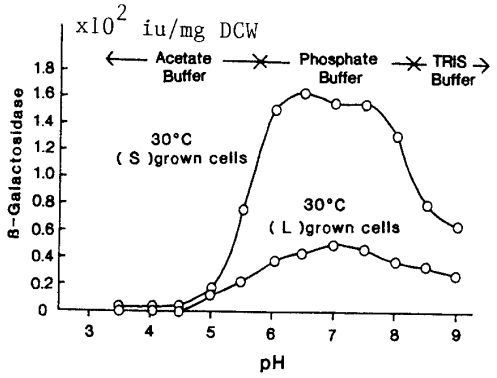

Fig.4. pH profile of β- galactosidase activity of the cells grown at 30°C, (L) with lactose and (S) without lactose

Discussion

The distinction of psychrophilic microorganisms from psychrotrophic ones is considered to be rather conventional than theoretical and it requires the observation for much prolonged time of the microbial survival with serial transplanting of them on the suitable media. So the classification of the microorganisms isolated in the present study is very much of temporary nature.

Concerning to the enzymic activity observed at the sub-zero degree temperature, particularly in the absence of glycerol is quite difficult to accept as a fact. The question whether the enzyme can really work even in the frozen environment presents a lot of yet unsolved problems. After repeated scrutiny a most plausible reason to answer the question has been recently conceived. That is inadequacy of the procedure to stop the enzymic reaction. The pH checked after the addition of Na_2CO_3 was 9.5. The last experiment in the present study shows that the enzyme has an appreciable amount of activity at pH 9.0.

So the time-consuming development of color after the enzyme reaction conducted at the low temperatures could be attributed to the residual activity working at the room temperature.

The result of the present study was presented at the annual meeting of the Society of Fermentation Technology, Japan on Nov. 15, 1990, and the further confirmation on this point will be made in the near future.

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