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Identification and Salt Requirement of Halophilic Bacteria isolated from Korean Salt-Fermented Sea Foods.

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한국 염장 발효식품으로 부터 분리한 호염성 세균의 분리·동정 및 염요구성

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Moderate halophilic bacteria isolated from several kinds of salted and fermented sea foods (jeotkal) collected from commercial market were identified and investigated on their salt requirements. It was confirmed that the isolates were dominantly moderate halophiles according to their NaCl requirement to grow. And their salt requirements in their growth have been examined for Na, K, Ni and Mg ions. Among them, the most dominant and distinctive three strains in protease production have been examined and two of them identified to be halophilic *Flavobacterium* sp., and the other one to be halophilic *Pseudomonas* sp.. Their optimum growth was observed at 30°C and at 10 percent of NaCl.

The existance of microorganisms growing well at high concentration of NaCl was reviewed by Flannery(1), although the growth of most microorganisms is inhibited in the presence of high concentration of salts.

Gibbons(2) difined that halophiles are organisms that need more than 3% salt for growth but the critical concentration of defining halophiles is rather difficult to determine.

In Korea, various kinds of salted and fermented sea foods were traditionally manufactured and consummed. It was thought that these salted foods were fermented by many kinds of halophiles in the presence of considerably high concentration (about 10%) of NaCl.

In order to examine physiological properties of these halophiles, isolations and characterizations of halophilic bacteria were carried out from Korean salted and fermented seafoods collected in Seoul and Kwangju city, and these isolates were examined on salt requirements.

Materials and Methods

Materials

Yeast extract, casamino acid and other media were products of Difco, U.S.A., and all other reagents of analytical grade were purchased from Sigma, U.S.A. Halophilic bacteria were isolated from local salted and fermented sea foods (jeotkal) in Seoul and Kwangju-city and provided in this study.

Methods

Isolation of halophilic bateria; Small amount of

Key words: Halophilic bacteria, Jeotkal (Korean salt-fermented sea food)
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salted and fermented sea food was suspended in sterilized saline water containing 10% NaCl. An aliquot of solution was spread on Sehgal and Gibbons agar medium(3) (SGM) or nutrient agar plate containing 10% NaCl. The plate was incubated at 30°C for several days. Single colonies appeared were isolated and transferred on SGM agar plate.

Cultivation of isolated strains; Isolated colony was inoculated into 100 m*l* Erlenmyer flask containing 20 m*l* SGM of various concentrations of salts and cultured on reciprocal shaker (150 strokes per min.) at 30°C.

Measurement of cell growth

The cell growth was determined by measuring turbidity of cells using turbidometer (Hf instruments Model DRT 100D, Fort Myers, Florida, U.S.A.) Turbidity values at ranges between zero and 1400 are equal in proportion to zero and 1.4 mg dry cell weight per m*l* of the halophilic bacteria used.

Identification of isolated strains

Morphological, biochemical and cultural properties of isolated strains were investigated according to the methods described by Cappuccimo and Sherman(4) and the strains were identified by Bergey's Manual of Determinative Bacteriology(5).

GC content of the isolated halophilic bacteria were determined by the method of Marmur *et al.*(7), using UV/VIS spectrometer, Gilford 2600.

GC content (mol% GC) = (Tm − 69.3)/0.41
(Tm: midpoint of melting temperature)

Results and Discussions

Isolation, identification of halophilic bacteria

To study the halophilic bacteria in Korean foods, fifteen kinds of salted and fermented sea foods (jeotkal) were collected from areas of Kwangju-city and Seoul.

Several moderate helophilic organisms were isolated from each samples on incubation in SGM containing 10% NaCl. (Table 1). Among them, strain H6 from myul-chi jeot II, strain H9 and H10 from sae-woo jeot I were able to grow well and to produce considerable amounts of proteolytic enzyme in SGM containing 10 to 20% NaCl at 30°C.

Table 1. Halophiles isolated from different sources

Sources of halophiles	No. of isolated strains
Salted and fermented anchovy (Myul-chi jeotkal) I	4
Salted and fermented anchovy (Myul-chi jeotkal) II	2
Salted and fermented shrimp (Sae-woo jeotkal) I	4
Salted and fermented shrimp (Sae-woo jeotkal) II	2
Salted and fermented intestine of Alaska(n) pollack (Chang-lan jeotkal)	5
Salted and fermented clame (Jo-gae jeotkal)	2
Salted and fermented sea-arrow (Col-tu-gi jeotkal) I	2
Salted and fermented sea-arrow (Col-tu-gi jeotkal) II	5
Salted and fermented horse- mackerel (Cheon-gang i jeotkal)	5
Salted and fermented cod-gill (Daegu-agami jeotkal)	3
Salted and fermented cod-egg (Daegu-al jeotkal)	3
Salted and fermented hickoryshad (Cheon-a jeotkal)	4
To-wha jeotkal	1
Don jeotkal	4
Pong-pu jeotkal	3

As shown in Table 2, all of these bacteria were gram negative and unable to ferment glucose, sucrose, fructose and other sugars examined. In order to identify gram negative halophilic bacteria, motility and pigment formation usually play the key roles of the characteristics(5). Strain H6 and H9 were non-motile and strain H10 motile. All of these three organisms formed orange or reddish pigments.

According to the identification of gram negative, yellow pigmented rods by Hayes *et al*(8), halophilic non-motile strains might be distinguished into two groups on the basis of their GC contents, Flavobacterim group 1 has low GC content (30-46%) and group II has high GC content. Thus, the strain H6 would appear to be the genus, *Flavo-*

Table 2. Morphological, cultural and biochemical characteristics of isolated moderate halophiles

Characteristics \ Strains	H6	H9	H10
Morphological characteristics			
Shape	rod	rod	rod
Size (μm)	1.5×1.0	1.5×1.0	1.25×1.0
Motility	−	−	+
Gram staining	−	−	−
Spore formation	−	−	−
Cultural characteristics			
Nutrient borth NaCl 10%	±	±	±
NaCl 20%	−	−	−
Sehgal & Gibbons medium (SGM)			
NaCl 10%	+ +	+ +	+ +
NaCl 20%	+ +	+ +	+ +
Norberg & Hofsten medium			
NaCl 10%	+ +	+ +	+ +
NaCl 20%	+ +	+ +	+ +
Biochemical characteristics			
Oxidase	−	+	+
Catalase	+	+	+
Urease	−	−	−
H_2S formation	−	−	
Acid production			
Sucrose	−	−	−
Glacose	−	−	−
Galactose	−	−	
Fructose	−	−	
Lactose	−	−	
Gelatine liquefaction	+	+	+ +
Reduction of nitrate	−	−	−
Observation of colony			
Color	orange	reddish	reddish
Form	circular	circular	circular
Surface	smooth	smooth	smooth
Moles % G+C	46	68	37
Tentative identification	*Flavo-bacterium* Section I	*Flavo-bacterium* Section II	*Pseudo-monas* sp.

bacterium group I and the strain H9 to be the genus *Flavobacterium* group II. As the GC content of the strain H10 was examined as 37%, it would be similar to be a *Pseudomonas* sp. from gram staining, motility pigment formation and other characteristics.

Effects of salt concentrations on growth of halophilic bacteria isolated

In order to examine influences of various kinds

of salts on growth and salt tolerances, the cultures of halophilic bacteria isolated were grown in SGM containing different concentrations of NaCl, KCl, LiCl and $MgCl_2$. As shown in Fig. 1 and Fig. 2, halophilic bacteria strain H6 grows well at the range of 1.5M to 3.4M of NaCl concentration in the SGM, but not grow at less than 0.8M and at 4.3M (25%) of the salt, suggesting to be a moderate halophilic.

The growth of the organism at the concentration of 1.5M NaCl wàs able to be partially replaced by the addition of KCl at the same concentration, but the growth was not observed at the concentration of more than 2.5 KCl without NaCl. But both LiCl and $MgCl_2 \cdot 6H_2O$ was unable to use in place of NaCl for the growth of the moderate halophilic bacteria.

It was reported by Onishi(6) that moderate halophile, *Acinetobacter* sp. 204-1 was grown well in SGM containing either NaCl below 3M or KCl lower than 1M(6). The cell growth of this strain was inhibited by higher concentration of NaCl or KCl, and not occured in the absence of NaCl, KCl. The strain H6 isolated in this study required the same amounts of NaCl or KCl for growth as *Acinetobacter* sp. 204-1 which did not form the pigment, and it is likely that strain H6 could be also belonged to

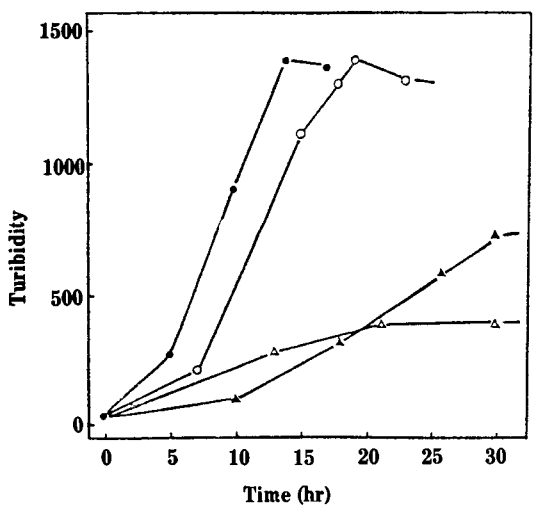

Fig. 1. Effects of various kinds of salts on growth of halophilic bacterium, strain H6.
●; 1.5 M NaCl ○; 1.5 M KCl △; 1.5 M Licl ▲; 1.5 M $MgCl_2$

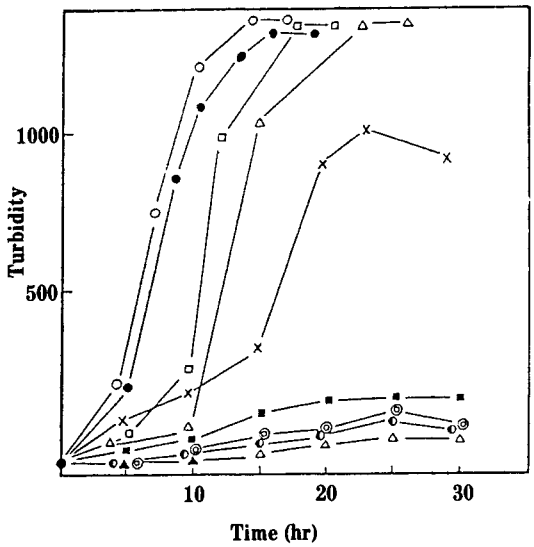

Fig. 2. Growth curve of moderate halophile H6 at different concentration of NaCl and KCl.
without NaCl and KCl; ◐KCl, ●; 1.5 M, ■; 2.5 M, ▲; 3.5 M, NaCl, ×; 0.85 M, ○; 1.5 M,□; 2.5 M,△; 3.5 M, ◉; 4.3 M

moderate halophile according to above characteristics.

When halophilic bacteria are exposed to hypotonic solution, then cells are lyzed. To prevent the lysis of cells, Li ion was recognized to be effective as well as Na ion and K ion(9). However, it should be emphasized that Li ion could not replace for Na ion in the growth of the strain H6 in the present experiment.

Effect of temperature on growth

Fig. 3 illustrates the growth patterns for the strain 6 at different incubation temperatures. The organism grew well at the range of 25 °C to 40 °C, but not at 50 °C, while extreme halophiles isolated from saltern grew well at 50 °C, (data not shown). Optimum growth of most moderate halophilic bacteria isolated here was observed at 30 °C in the case of pH7.

요 약

염장 식품인 젓갈류 15종류에서 호염성 세균을 분리하여 그들이 성장할 수 있는 NaCl의 농도에 따라, 5-10% NaCl에서 잘 자라는 18균주와 10-20% NaCl

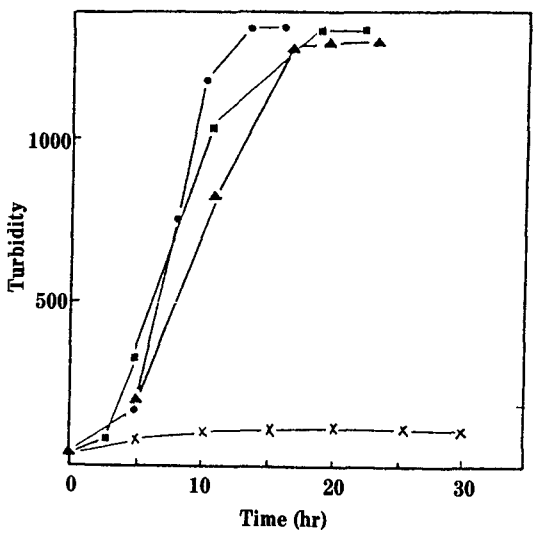

Fig. 3. Growth curve of moderate halophile H6 at different temperature
□; 25°C ●; 30°C▲; 40°C ×; 50°C

에서 더욱 잘 자라는 35균주를 구분하여, 이들을 moderate halophiles로 분류하였다.

분리한 moderate halophiles중에는 *Flavobacterium*-group I과 II 그리고 *Pseudomonas* sp.에 속하는 균주들이 있으며 이들은 30℃에서 10% NaCl을 첨가했을 때 가장 잘 성장하였다.

Na이온 대신 K이온을 대치시켰을 때 1.5M에서는 대치가 가능하였으나 그보다 높은 농도인 2.5M 내지 3.5M에서는 대치될 수 없음을 확인하였고 Na이온 대신 Li이온이나 Mg이온은 생장에 적합하지 않았다.

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