# SALT TOLERANCE OF BACTERIA IN ESTUARINE SEDIMENTS

by

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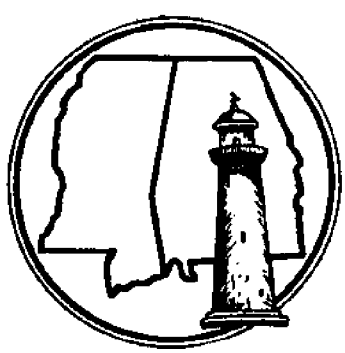

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# Salt Tolerance of Bacteria in Estuarine Sediments

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## Introduction

Variations in salinity may contribute to population shifts in bacterial species, with respect to prevalent types, and these shifts might be used as an indication of potential large scale variations in marine food chains. Identification of microbial species could also be a reliable method for monitoring subtle differences in edible fish populations in the marine estuaries. Specific sensitivity to various industrial pollutants exhibited by certain organisms might be useful in monitoring low levels of toxic substances which invade sampling areas at intervals throughout the year. Such a project could and should be expanded to include bacteria, algae, and other free-living marine organisms to increase the validity of the tests. In other words, a catalogue of the entire ecosystem might be carried out at the level of sediment biomass, with specific variation markers to better define a reproducible bioassay.

## Objective of Study

There were two objectives to this study; one, to acquaint a potential graduate students with research in marine microbiology, and two, to accumulate information on the metabolic requirements of microbial components of marine sediments.

Scientifically this was designed to determine sea salt requirements of bacteria found in marine sediments of coastal waters. More specifically, the questions dealt with were 1) Are there any obligate halophiles present and 2) what salt requirements are there for other organisms in the biosystem.

## Materials & Methods

### Rila Salts

Rila Sea Salts Mix[1] was used in the laboratory study to achieve appropriate salinity.

### Culture Media

Media used in this study was obtained from Difco[2]. The media included Tryptic Soy Agar (TSA) and Tryptic Soy Broth. The media were prepared in the following manner.

a) 35 parts/thousand Rila Salts with TSA - 1000 ml Rila Sea Salts Solution (at 35 ppt) + 40 grams dehydrated TSA

b) 15 ppt Rila Salts with TSA - 428.5 ml Rila Salts solution (35 ppt) + 571.5 ml distilled water + 40 grams dehydrated TSA

c) 0 ppt Rila Salts with TSA - 1000 ml distilled water + 40 grams dehydrated TSA

d) 35 ppt Rila Salts with TSA broth - 1000 ml distilled water + 30 grams dehydrated TS broth

e) 15 ppt Rila Salts with TSA broth - 428.5 ml Rila Salts Solution (35 ppt) + 57.5 ml distilled water + 30 grams dehydrated TS broth

f) 0 ppt Rila Salts with TSA broth - 1000 ml distilled water + 30 grams dehydrated TS broth

[1]Rila Products, Teaneck, New Jersey

[2]Difco Laboratories, Detroit, Michigan

Isolation procedures

Ten grams of a marine sediment sample was run through serial dilutions 0.1 ml spread plated on 4 plates 35 ppt salt TSA for each dilution and incubated at 20°C for four days. These plates were replicated and growth characteristics for salt were observed. Colonies were picked from the original plates (except in one case, where one was picked from a replica plate) and placed in sterile water & sand in a screw cap test tube. The tubes were mixed thoroughly and then plated for isolation on 35 ppt salt TSA & streaked on 35 ppt salt TSA slants. Once isolated, these colonies were placed in TS broths of 35, 15, and 0 ppt salt from the plate or slant (whichever gave the best isolation). One was obtained from the original plate and one from a replica plate. The broths were incubated at 20° for 3 days, whereupon, they were refrigerated for storage.

Gradient plating

Aliquots of 0 ppt or 35 ppt salt TSA were poured into a sterile petri dish, the plate slanted to produce an incline in the agar inside the plate and the agar allowed to harden overnight. The next day the opposite concentration of salt (35 ppt if 0 or 0 if 35) was poured after allowing the liquid agar to cool to just before solidification over the first layer to make a flat agar surface and the agar allowed to harden. Once hardened, the plates were streaked with three organisms horizontal to the gradient slant (see Set 1, #4) and incubated at 20°C for 3 days.

Plates should be streaked as soon as possible after the overlay hardens to prevent diffusion between the two agars. Bottom halves should also be marked as to which way the gradient goes after the first layer is poured.

Spread plating

0.1 ml of a serial dilution from 10 grams marine soil was placed on 35 ppt Rila salt TSA and spread with glass rods. In the first three series a 1 ml pipette was used and in the last a 100 ul Eppendorf pipette was employed for increased reproducibility. Organisms were streaked on 35 ppt salt TSA plates and colonies picked to TSA slants for storage.

Replica plating

After 0.1 ml of a serial dilution was spread plated upon 35 ppt Rila salts TSA and grown out, plates with between 5-40 colonies were chosen to be replica plated. This process involved utilizing a round wooden block of slightly less diameter than the petri dish with a velvet bottom. This was placed on the original plate and colonies imprinted on the velvet then transferred to a set of appropriate dishes. The plates were marked to obtain proper orientation for the replica procedure and also for comparing plates after incubation.

One problem incurred with this method was that colonies were larger on replica plates due to spreading out of colony when the velvet was pressed on the plates. Also, higher colonies may not allow the velvet to reach smaller, lower colonies so they may not appear on replica plates. One should not press the velvet on the original plate with too much force since this will cause colony spreading and therefore confuse the results.

Photography

Photographs of plates were taken with a Pentax K1000 35 mm camera to illustrate replica plating, gradient plates, and basic colonial merphology.

## Numbering System

The numbering system is as follows. The letter represents the series of plates as described in the results. The 1st number refers to the reciprocal of the dilution. The subscripts refer to the individual plates. R refers to a replica plate.

## Definitions

Obligate halophiles are absolute salt requiring organisms as determined by a 0 to 35 ppt gradient. Semi-obligate halophiles prefers elevated salt levels but is facultative with respect to growth. Facultative organisms grew under all of the salt concentrations employed semi-non obligate halophiles will tolerate elevated salt levels but grow better at lower levels. Such determinations were based entirely on visual judgement.

## Results and Conclusions

I. A series - 10 grams soil from marine sediment sample (Jar #300) frozen for several years - run through dilution series - 0.1 ml spread plates on 4 plates 35 ppt salt TSA for each dilution

Plate counts

| | | |
|---|---|---|
| 1/1000 dilution | $^{3}A3_{1-4}$ | $^{4}$TNTC |
| 1/10,000 dilution | $^{5}A4_{1-4}$ | approx 200 colonies |
| 1/100,000 dilution | $^{6}A5_{1-26}$ | |
| | $A5_{2-14}$ | $\bar{x}$ - 23 |
| | $A5_{3-36}$ | s - 10.1 |
| | $A5_{4-16}$ | est microbes/g = $2.3 \times 10^{5} \pm 10.1$ |

| Replica plates | $A5_1R$ | $A5_2R$ | $A5_3R$ | $A5_4R$ | $\bar{x}$ | % |
|---|---|---|---|---|---|---|
| obligate halophiles | 2 | 0 | 1 | 2 | 1.25 | 5.0% |
| semi-obligate halophiles | 1 | 1 | 9 | 0 | 3.667 | 14.7% |
| facultative halophiles | 13 | 6 | 17 | 14 | 12.5 | 50.2% |
| semi-non obligate halophiles | 7 | 5 | 12 | 0 | 6 | 24.1% |
| no replicates | 3 | 2 | 1 | 0 | 1.5 | 6.02% |

$^{3}A^{3}$ refers to $10^{-3}$ dilution. 1-4 refers to plate numbers.

$^{4}$Too numerous to count

$^{5}A^{4}$ refers to $10^{-4}$ dilution

$^{6}A^{5}$ refers to $10^{-5}$ dilution

II. B series - same procedure as A (Jar #290)

Plate counts

| | | |
|---|---|---|
| 1/1000 dilution | $B3_{1-4}$ | TNTC |
| 1/10,000 dilution | $B4_1$ | approx 200 colonies; 2 species |
| $1/10^5$ dilution | $B4_2$ | 200-250 colonies; 3 species |
| | $B4_3$ | same as $B3_1$ |
| | $B4_4$ | same as $B3_1$ |
| | $^7B5_1$ | NG |
| | $B5_2$ | 2 $\bar{x}$ - 3.5 |
| | $B5_3$ | 8 s - 3.4 |
| | $B5_4$ | 4 est microbes/g $3.5 \times 10^4 \pm 3.4 \times 10^4$ |

Replica plates

$B^4_{1,3,4}$ - all appear as facultative halophiles

$B^4_2$ - mostly facultative halophiles with a few semi-obligate halophiles

III. C series - 10 grams of fresh sediment from Bay St. Louis

- run through same procedure as above

Plate counts

| Dilution: | $1/10^5$ | $1/10^6$ | $1/10^7$ | $1/10^8$ |
|---|---|---|---|---|
| | $C5_1$-1 | $C6_1$-NG | $C7_1$-2 | $C8_1$-NG |
| | $C5_2$-NG | $C6_2$-NG | $C7_2$-NG | $C8_2$-NG |
| | $C5_3$-NG | $C6_3$-3 | $C7_3$-NG | $C8_3$-1 |
| | $C5_4$-NG | $C6_4$-NG | $C7_4$-4 | $C8_4$-NG |

$^7$No growth

Repica plates - not done

IV. D series - same procedure as C only using different dilutions and all 3 salt concentrations (0, 15, & 35 ppt) on TSA

| Plate counts | | Dilution | | | | | |
|---|---|---|---|---|---|---|---|
| salt conc. | Plate | D3 1/1000 | D4 $1/10^4$ | D5 $1/10^5$ | D6 $1/10^6$ | D7 $1/10^7$ | D8 $1/10^8$ |
| 35 ppt | 1 | 17 | NG | 2 | | | |
| | 2 | 15 | 3 | 8 | | | |
| | 3 | 13 | 6 | 1 | | | |
| | 4 | 12 | 6 | 1 | | | |
| 15 ppt | 1 | | | 3 | 1 | 2 | |
| | 2 | | | 2 | 1 | NG | |
| | 3 | | | 3 | NG | NG | |
| | 4 | | | 2 | 2 | NG | |
| 0 ppt | 1 | | | 11 | TNTC | NG | NG |
| | 2 | | | 7 | 1 | NG | NG |
| | 3 | | | 3 | 3 | 1 | NG |
| | 4 | | | 5 | 1 | 2 | NG |

*NR - not run

| | Original - 35 ppt TSA | | | | | Original - 0 ppt | | |
|---|---|---|---|---|---|---|---|---|
| Replica plates | $D3_1$ | $D3_2$ | $D3_3$ | $D3_4$ | % | $D5_2$ | $D5_3$ | % |
| obligate halophiles | 0 | 0 | 0 | 0 | % | 0 | 0 | 0% |
| semi-obligate halophiles | 0 | 0 | 0 | 0 | % | 0 | 0 | 0% |
| faculatative halophiles | 7 | 8 | 11 | 8 | 57.6% | 6 | 5 | 50% |
| semi-non obligate halophiles | 7 | 6 | 2 | 1 | 27.1% | 0 | 1 | 4.5% |
| obligate non halophile | 0 | 0 | 0 | 0 | 0% | 0 | 1 | 4.5% |
| no replication | 3 | 2 | 0 | 4 | 13.56% | 1 | 8 | 41% |

est # microbes/g

from 0 ppt D5 $\bar{x} = 6.5$ $S = 3.42$ - $6.5 \times 10^4 \pm 3.42 \times 10^4$

from 15 ppt D5 $\bar{x} = 2.5$ $S = .578$ - $2.5 \times 10^4 \pm 5.78 \times 10$

from 35 ppt D3 $\bar{x} = 14.25$ $S = 2.217$ - $1.425 \times 10^3 \pm 2.22 \times 10^2$

Average - $3.0475 \times 10^4$ microbes/g

The A series replication of this study is described first. The plates of the $1/10^5$ dilution gave a reasonable number of colonies to replicate and also several had varying degrees of salt tolerance. This shows that possibly this marine sediment sample was from an area of high salt content that remained fairly stable. Replica plates turned out well and the $A5_3$ series is displayed in accompanying photographs (see Set 1, #1-4). Also certain colonies with varying salt requirements were isolated and further characterized.

The B series, however, did not produce workable replica plates. Three plates of the dilution that gave approx 200 colonies were replicated with 1 plate of the next highest dilution. As most of the colonies seemed to be of only three types, little variety was noted as two were facultative halophiles with one semi-facultative halophile. The semi-obligate halophile was isolated for further study.

The C series involved fresh sediment sample from Bay St. Louis. The salt concentration in this bay varies with the tides and inflowing rivers and may also vary at different depths of the bay. The results of this test were not consistant and were disregarded as the dilution was too high for plating on the 35 ppt salt TSA.

The D series also involved fresh sediment samples from Bay St. Louis. This series involved plating dilutions on 0, 15, & 35 ppt salt TSA, as outlined in Table I. Plate counts of this series gave erratic results and values determined for total population/g should be ignored. This variation is probably due to sample variation when preparing the dilutions. However, six replicable plates were workable and replicated, 2 from the 1/100,000 0 ppt salt TSA, and 4 from the 1/1000 35 ppt salt TSA. The replica results however gave only facultative halophiles which indicates that the organisms present in the marine sediment can withstand the varying salinity of Bay St. Louis.

| Origin | | Original colony description | Original thoughts on salt requirement |
|---|---|---|---|
| 1 | $A5_1$ | white translucent | facultative hal. |
| 2 | $A5_1$ | small white | semi-non-obligate hal. |
| 3 | $A5_1$ | small yellow | obligate halophile |
| 4 | $A5_1$ | medium yellow | obligate halophile |
| 5 | $A5_2$ | small white | semi-non-obligate hal. |
| 6 | $A5_2$ | large white | facultative hal. |
| 7 | $A5_2$ | small white | unknown (no replica growth) |
| 8 | $A5_3$ | small white | facultative hal. |
| 9 | $A5_3$ | small yellow | semi-obligate halophile |
| 10 | $A5_3$ | small yellow | facultative halophile |
| 11 | $A5_3$ | small white | semi-non-obligate halophile |
| 12 | $A5_4$ | small yellow | obligate halophile |
| 13 | $A5_4$ | large white | facultative halophile |
| 14 | $A4_1$ | medium yellow-orange | obligate halophile |
| 15 | $A5_3$ | small white | semi-non-obligate hal. |
| 16 | $B3_2R(15)$ | small yellow | semi-obligate halophile |

The above table gives the first impressions of the colonies chosen for further testing. They were chosen for variety to give an all around view of the marine organisms. This does not mean that all species found in the sediment were tested. The organisms were placed in sterile screw cap tubes with sterile water & sand.

Results slants (35 ppt salt)

| Colony | |
|---|---|
| 1 | good growth |
| 2 | good growth |
| 3 | NG |
| 4 | slight growth |
| 5 | NG |
| 6 | good growth |
| 7 | medium growth |
| 8 | NG |
| 9 | NG |
| 10 | NG |
| 11 | NG |
| 12 | NG |
| 13 | medium growth |
| 14 | medium growth |
| 15 | NG |
| 16 | medium growth |

These slants were inoculated from the sterile water & sand screw cap tubes so in some cases not enough organisms were present for growth on the slants or perhaps the salinity inhibited growth. But if the organisms did grow they were then inoculated into the TS broths.

Origin of broths

| Colony | Source | Colony | Source |
|---|---|---|---|
| 1 | slant | 9 | plate isolation |
| 2 | slant | 10 | plate isolation |
| 3 | $A3_1$ | 11 | plate isolation |
| 4 | plate isolation | 12 | plate isolation |
| 5 | plate isolation | 13 | slant |
| 6 | slant | 14 | slant |
| 7 | slant | 15 | $A3_3R$ |
| 8 | plate isolation | 16 | slant |

Broths

Results from growth in the broths would be inconclusive as the salt tended to drop out of solution giving a high salinity at the bottom with little at the top in the 35 ppt salt broth. They were best used as a source of the organisms.

Gradient plate results:

| Colony | 1st (0/35) | 2nd (0/35) | 3rd (0/35) | ppt of origin broth |
|---|---|---|---|---|
| 1 | facultative hal | facul. hal | facul. hal | 0 |
| 2 | semi-non-obligate | facul. hal | facul. hal | 0 |
| 3 | NG | facul. hal | semi-non-obligate hal | 0 |
| 4 | | facul. hal | inconclusive | 0 |
| 5 | No 0 ppt overlay | facul. hal | semi-non-obligate hal | 0 |
| 6 | | facul. hal | facultative hal | 0 |
| 7 | semi-non-obligate hal | semi-non-obligate hal | facul. hal | 35 |
| 8 | semi-non-obligate hal | semi-non-obligate hal | facul. hal | 15 |
| 9 | NG | facultative hal | ---- | -- |
| 10 | NG | facul. hal | NG | 0 |
| 11 | NG | facul. hal | facul. hal | 15 |
| 12 | NG | facul. hal | semi-obligate hal | 15 |
| 13 | facultative hal | facul. hal | facul. hal | 0 |
| 14 | NG | facul. hal | facul. hal | 0 |
| 15 | NG | facul. hal | facul. hal | 0 |
| 16 | not done | facul. hal | facul. hal | 15 |
| origin | screw top tubes | TSA broths | TSA broths | |

The first attempt at gradient plates was an inexperienced, amaturish one. One problem incurred was inoculating the plates from the sterile distilled water and sand tubes which did not have enough organisms in most cases as illustrated by all the No growths. So these results should be ignored.

The second attempt has a greater reason for being ignored. The gradient plates were prepared a week and a half before use. This time allowed the agars to diffuse together giving an overall salt concentration of between 15-20 ppt.

The third attempt was the best by far. An attempt was made to inoculate the plates from the same salt conc. but several broths were contaminated. These plates were inoculated once the second layer had hardened so lessing any chances for diffusion. But the results from inoculating on 35 ppt vs 0 ppt seemed to give better results than the gradient plates.

The following isolates were stored for further studies:

| Colony | Salt (ppt of broth inoculated from) | results | gram stain |
|---|---|---|---|
| 1 | 35 | faculatative halophile | - cocci |
| 2 | 35 | inconclusive | - cocci |
| 3 | 35 | semi-non-obligate halophile | - cocci |
| 4 | 35 | semi-obligate halophile | - rods |
| 5 | 35 | semi-non-obligate halophile | + rods |
| 6 | 35 | facultative halophile | - chained diplococci |
| 7 | 35 | facultative halophile | - chained diplococci |
| 8 | 15 | semi-obligate halophile | - diplococci |
| 9 | -- | ---- | ---- |
| 10 | 35 | facultative halophile | - diplococci |
| 11 | 15 | facultative halophile | + filametious/-spore sta |
| 12 | 15 | obligate halophile | - cocci |
| 13 | 0 | facultative halophile | - chained diplococci |
| 14 | 0 | semi-obligate halophile | - chained diplococci |
| 15 | 35 | facultative halophile | - diplococci |
| 16 | 15 | facultative halophile | + rods |

This is a comparison study between growth on 0 ppt salt TSA & 35 ppt salt TSA. Only one really obligate halophile was found in the entire study (#12). The rest either are salt tolerant or require a small amount of salt for optimal growth. The 35 ppt salt broth may have had some effect on the growth but this probably didn't occur as the salt dropped out of solution in these broths.

Gram staining of the organism gave a clue to their morphology, mostly gram-cocci or diplococci. Colony #1 was observed to be highly filamentious, making it possibly Bacillus or Clostridum, but this was contraindicated by the negative spore stain.

Pictures

Set 1 - A series 1/10,000 ($A5_3$)

original on 35 ppt salt TSA

1) original plate
2) 15 ppt. salt replica
3) 0 ppt. salt replica

Set 2 - D series (1/1000) 1st group ($D3_1$)

original - 35 ppt salt TSA

1) original
2) 0 ppt salt replica
3) 15 ppt salt replica
4) 35 ppt salt replica

Set 3 - D series (1/100,000) 2nd group ($D5_2$)

original - 0 ppt salt TSA

1) original plate
2) 0 ppt salt replica
3) 15 ppt salt replica
4) 35 ppt salt replica

Set 4 - Gradient plate

35 ppt left → 0 ppt right

1) 1st streak - colony #11 - facultative howl
2) 2nd streak - colony #12 - obligate halophile
3) 3rd streak - colony #13 - facultative hal

Set 5 - 0 vs 35 ppt - colony 12

obligate halophile

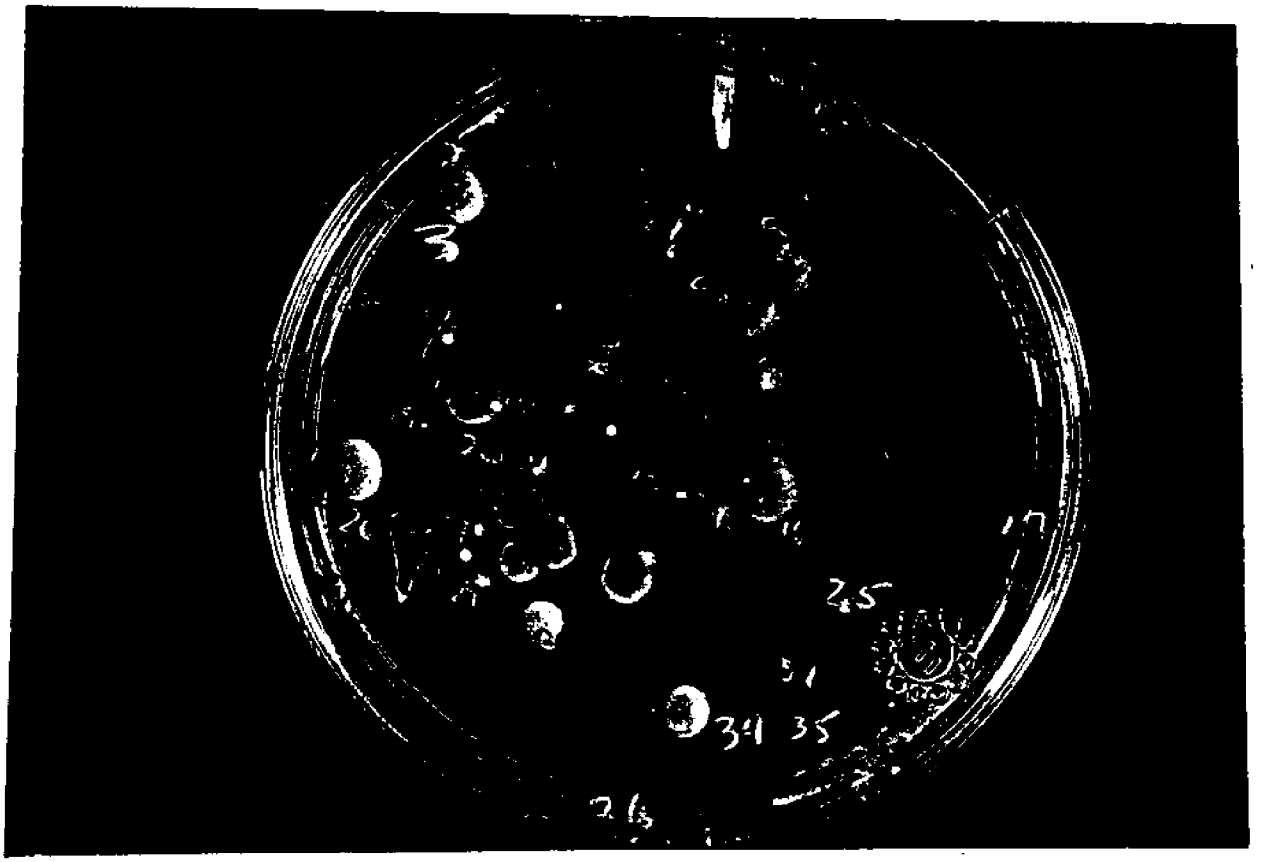
1) 0 ppt salt TSA
2) 35 ppt salt TSA

Set 6 - 0 vs 35 ppt - colony #1

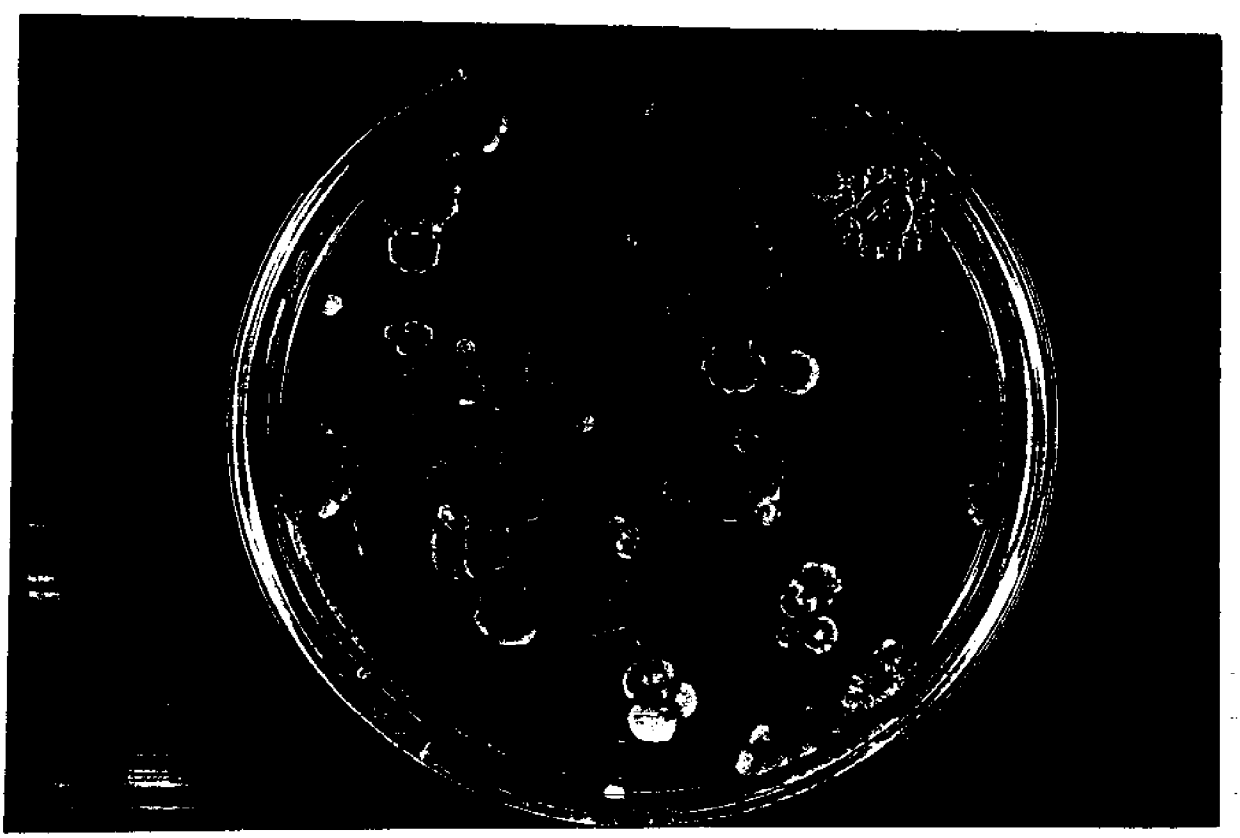
facultative halophile

1) 0 ppt salt TSA
2) 35 ppt salt TSA

| | |
|---|---|
| Set 7 - 0 vs 35 ppt - colony #5 | 1) 0 ppt salt TSA |
| | 2) 35 ppt salt TSA |

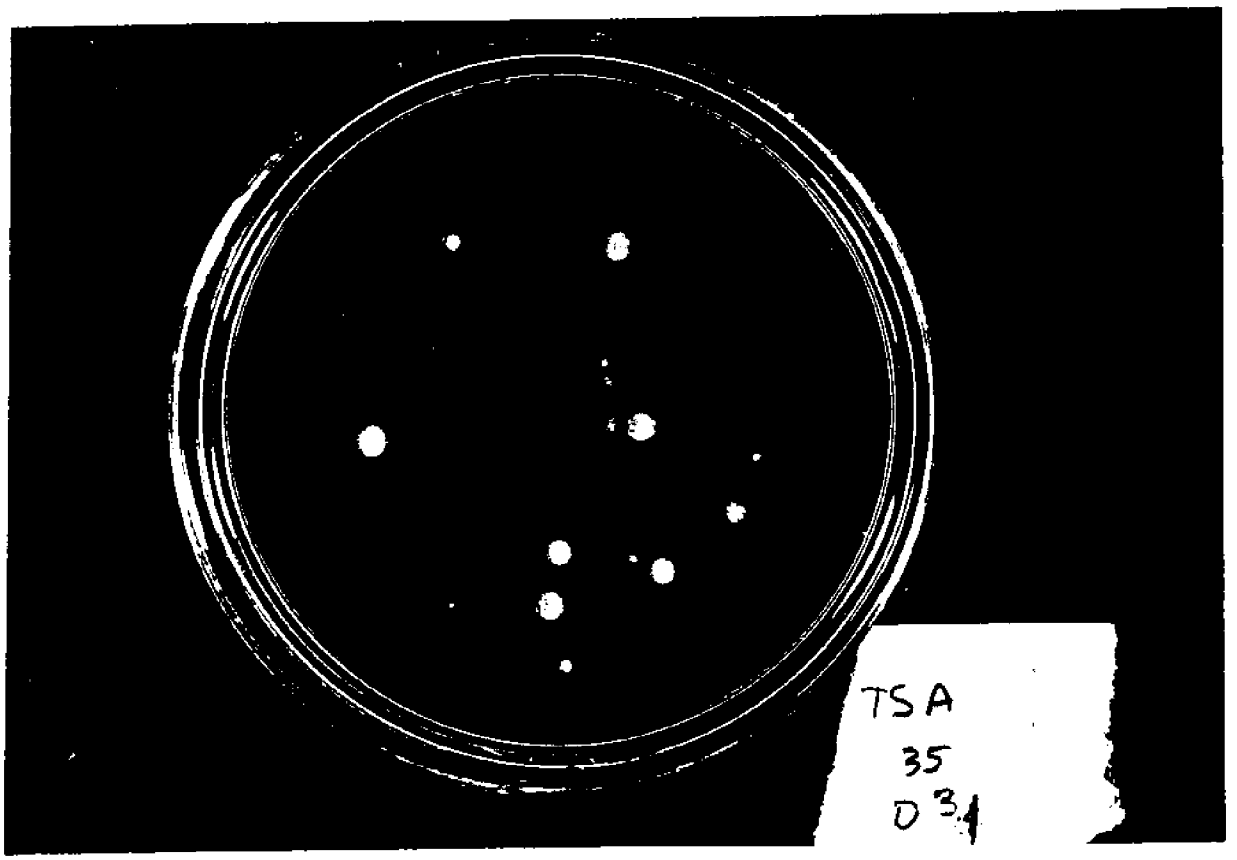



Set 1 - A series 1/10,000 ($A5_3$) original on 35 ppt salt TSA #1



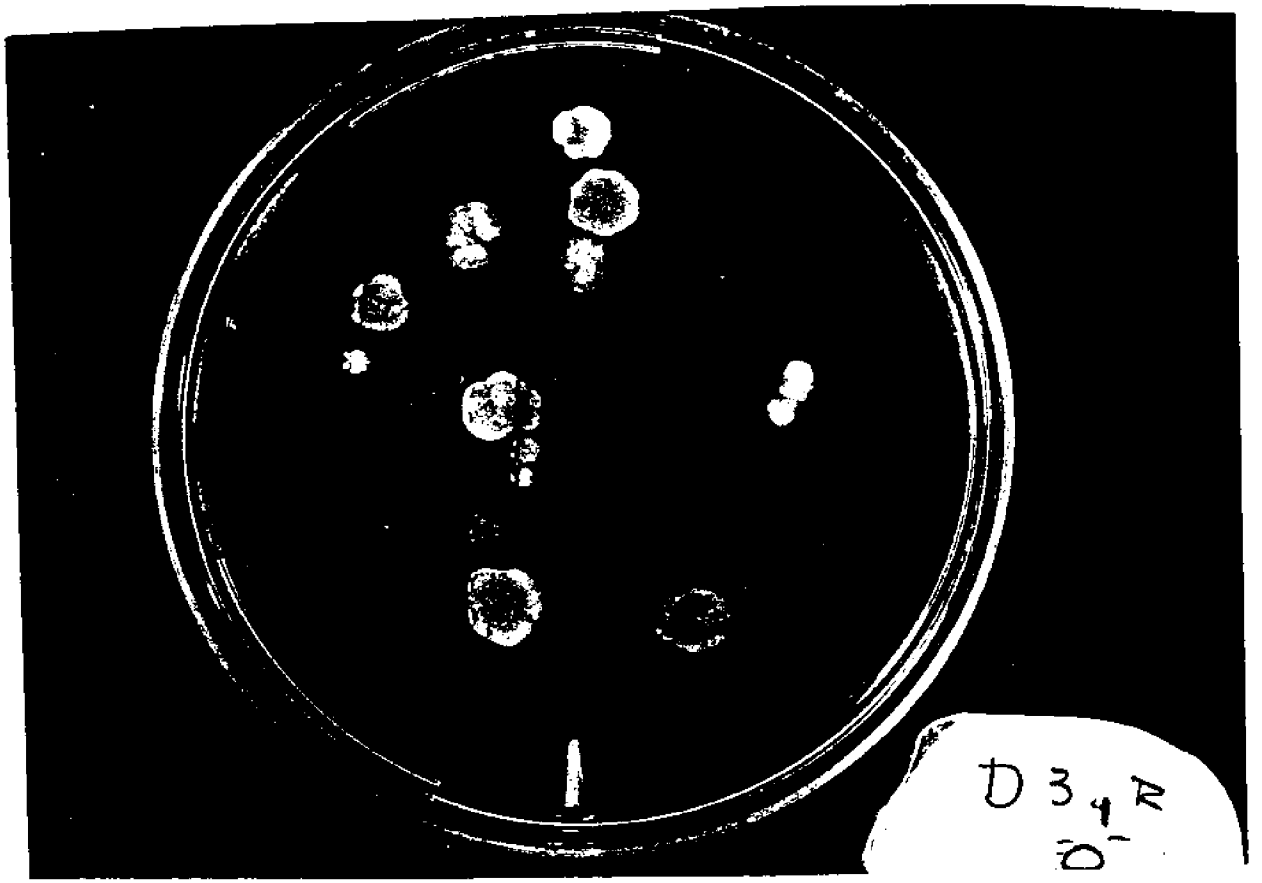
Set 1 - A series 1/10,000 ($A5_3$) original on 35 ppt salt TSA # 2


Set 1 - A series 1/10,000 ($A5_3$) original on 35 ppt salt TSA # 3

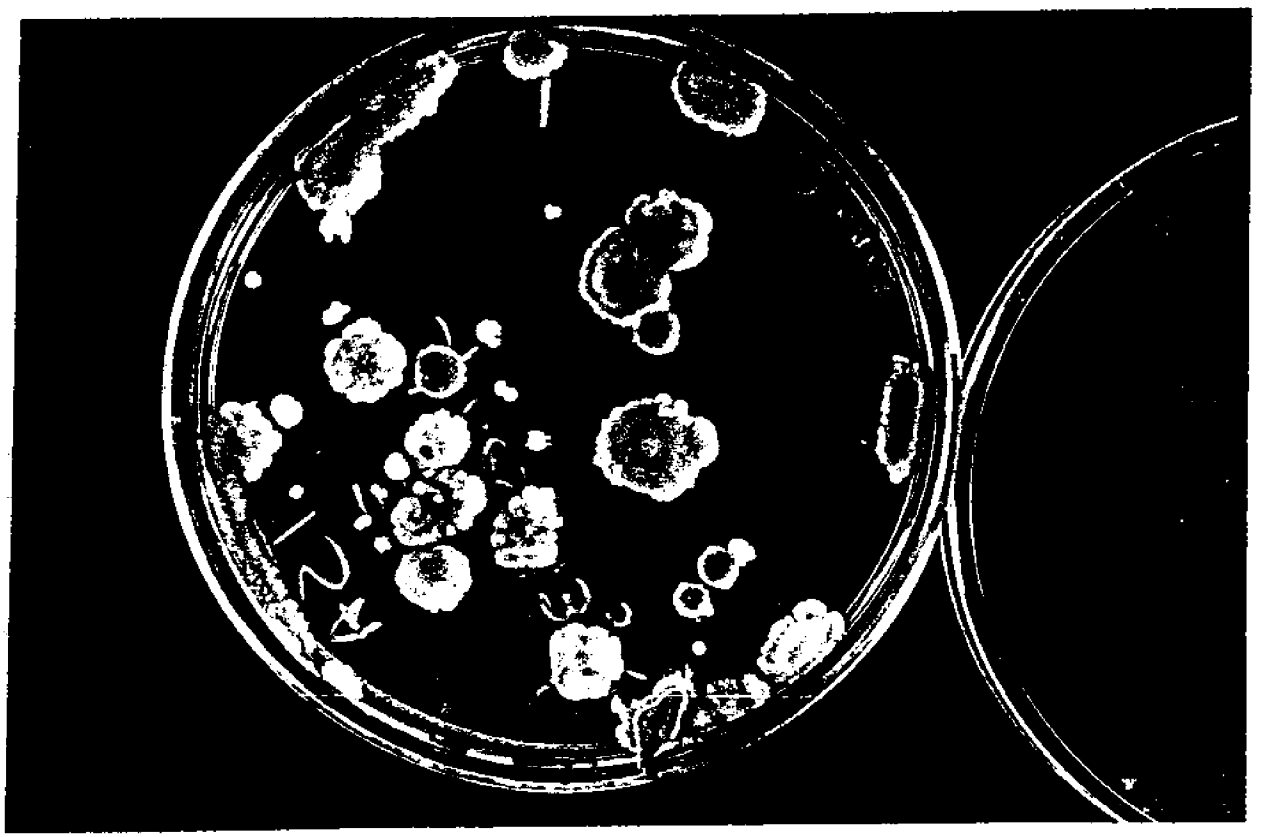



Set 2 - D series 1/1000 1st group ($D3_1$) original on 35 ppt salt TSA #1

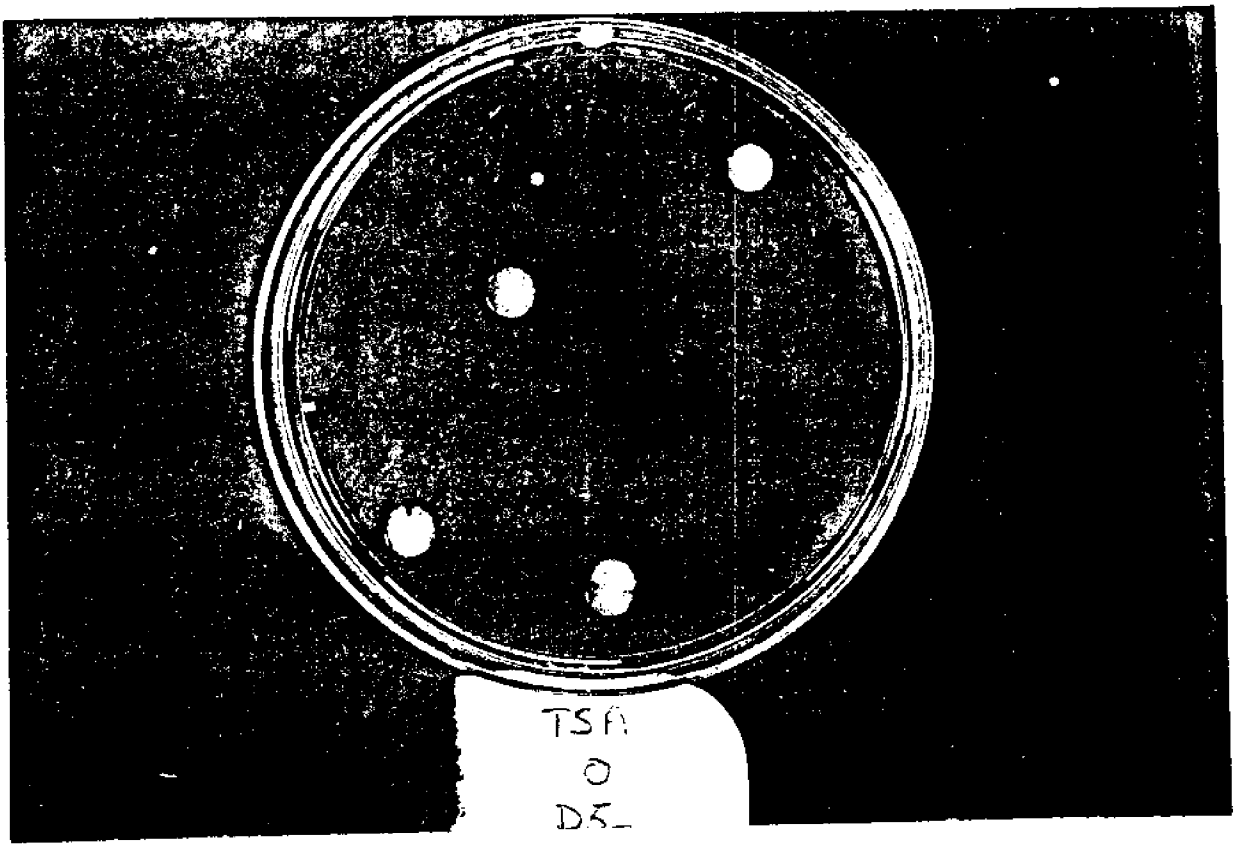
Set 2 - D series 1/1000 1st group ($D3_1$) original on 35 ppt salt TSA #2



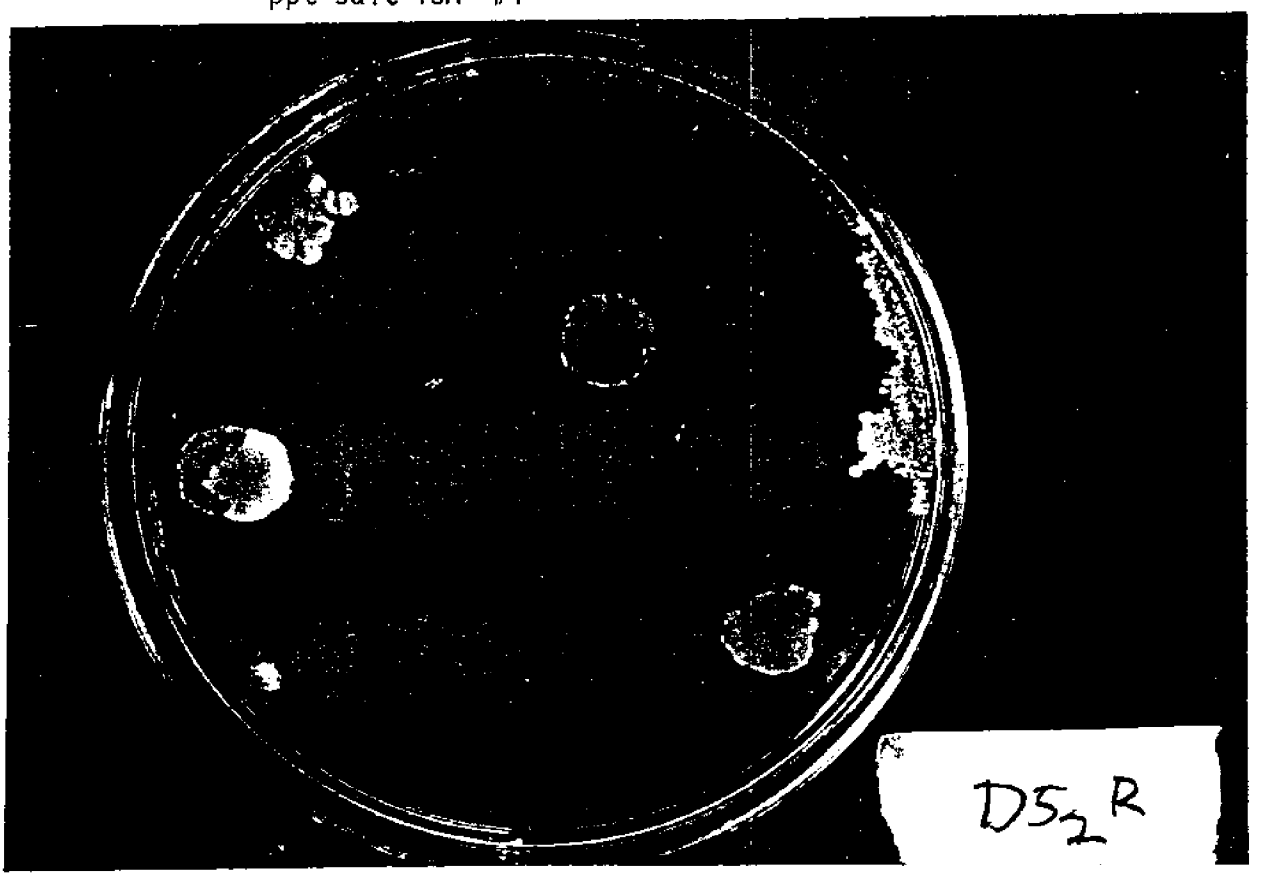

Set 2 - D series 1/1000 1st group ($D3_1$) original on 35 ppt salt TSA #3



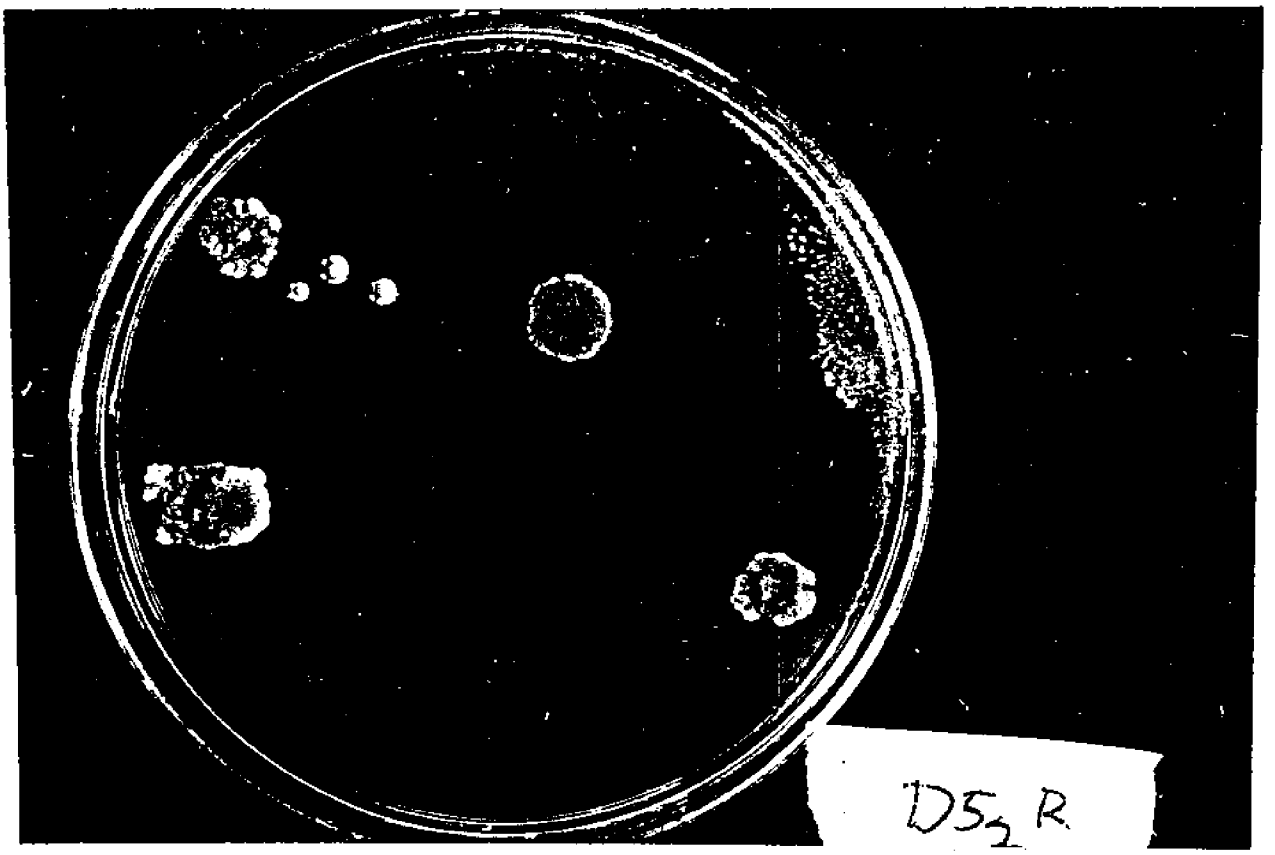
Set 2 - D series 1/1000 1st group ($D3_1$) original on 35 ppt salt TSA #4
(Plate 5)



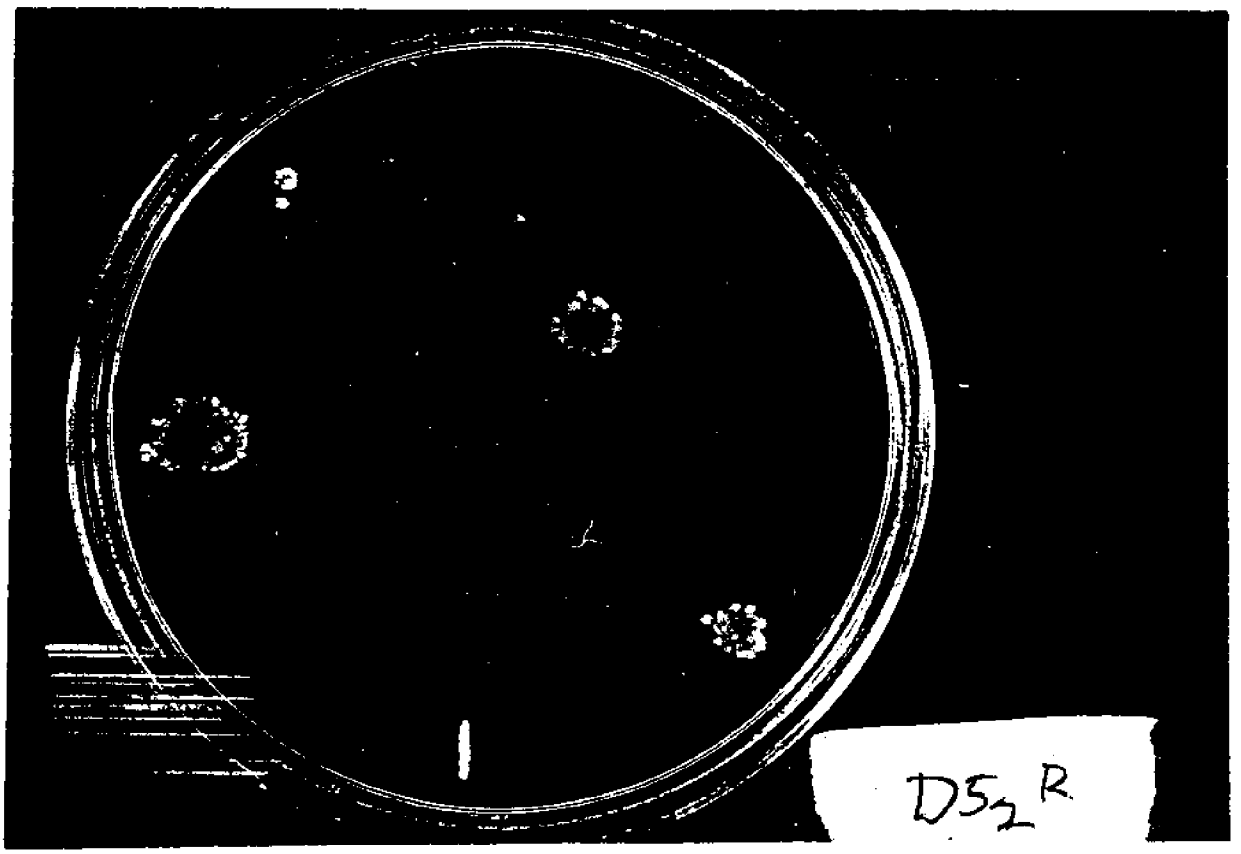

Set 3 - D series 1/100,000 2nd group ($D5_2$) original on 0 ppt salt TSA #1



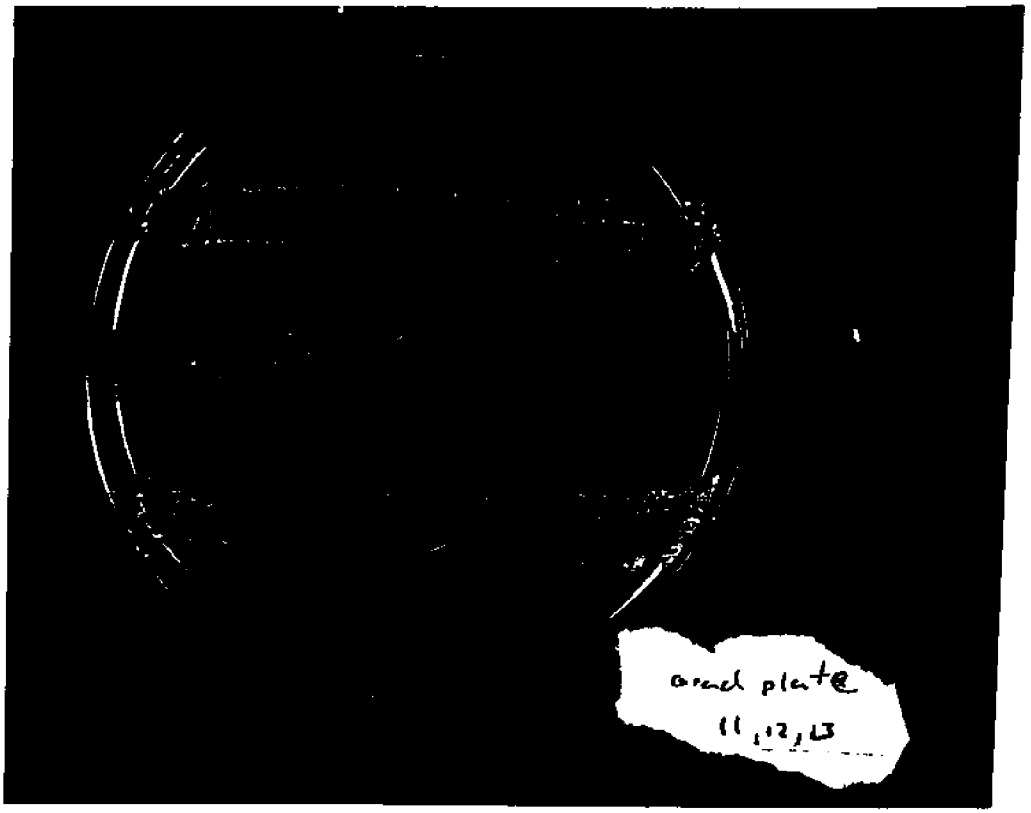

Set 3 - D series 1/100,000 2nd group ($D5_2$) original on 0 ppt salt TSA #2



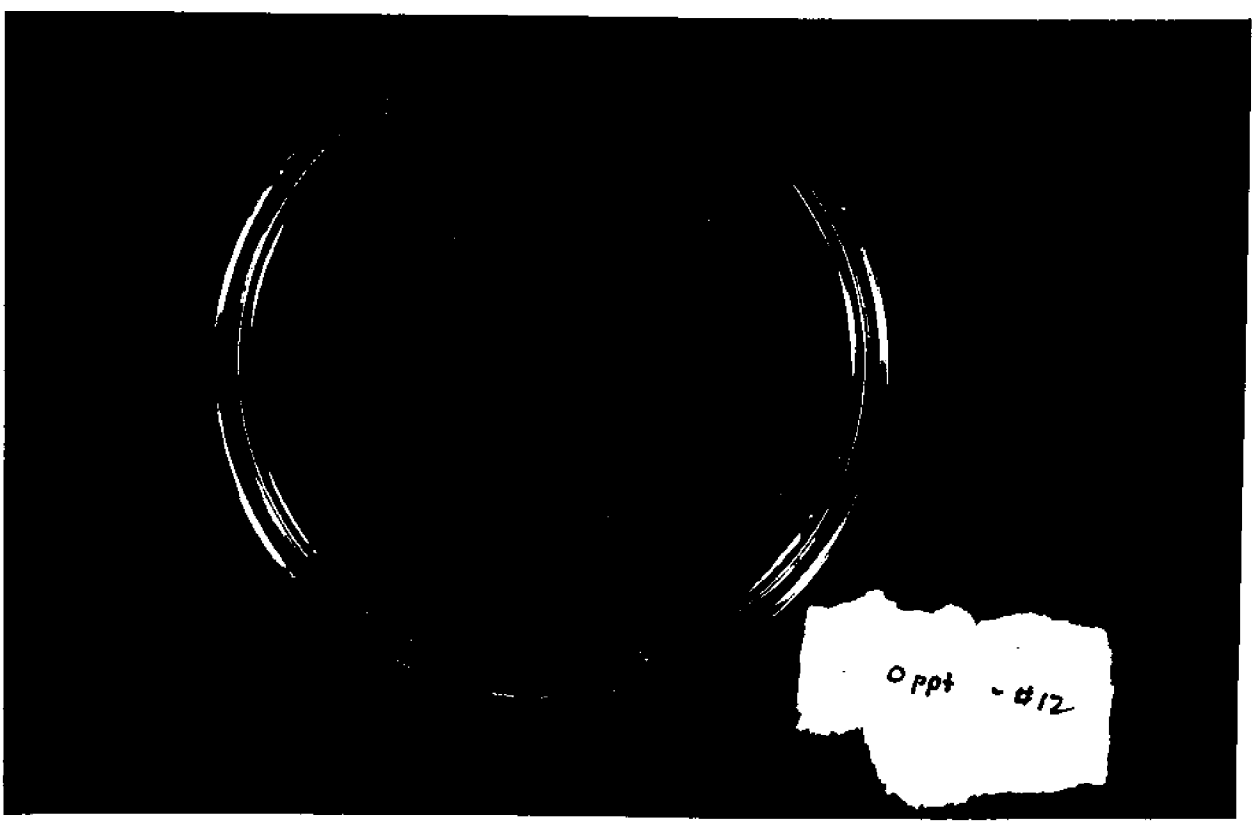

Set 3 - D series 1/100,000 2nd group ($D5_2$) original on 0 ppt salt TSA #3



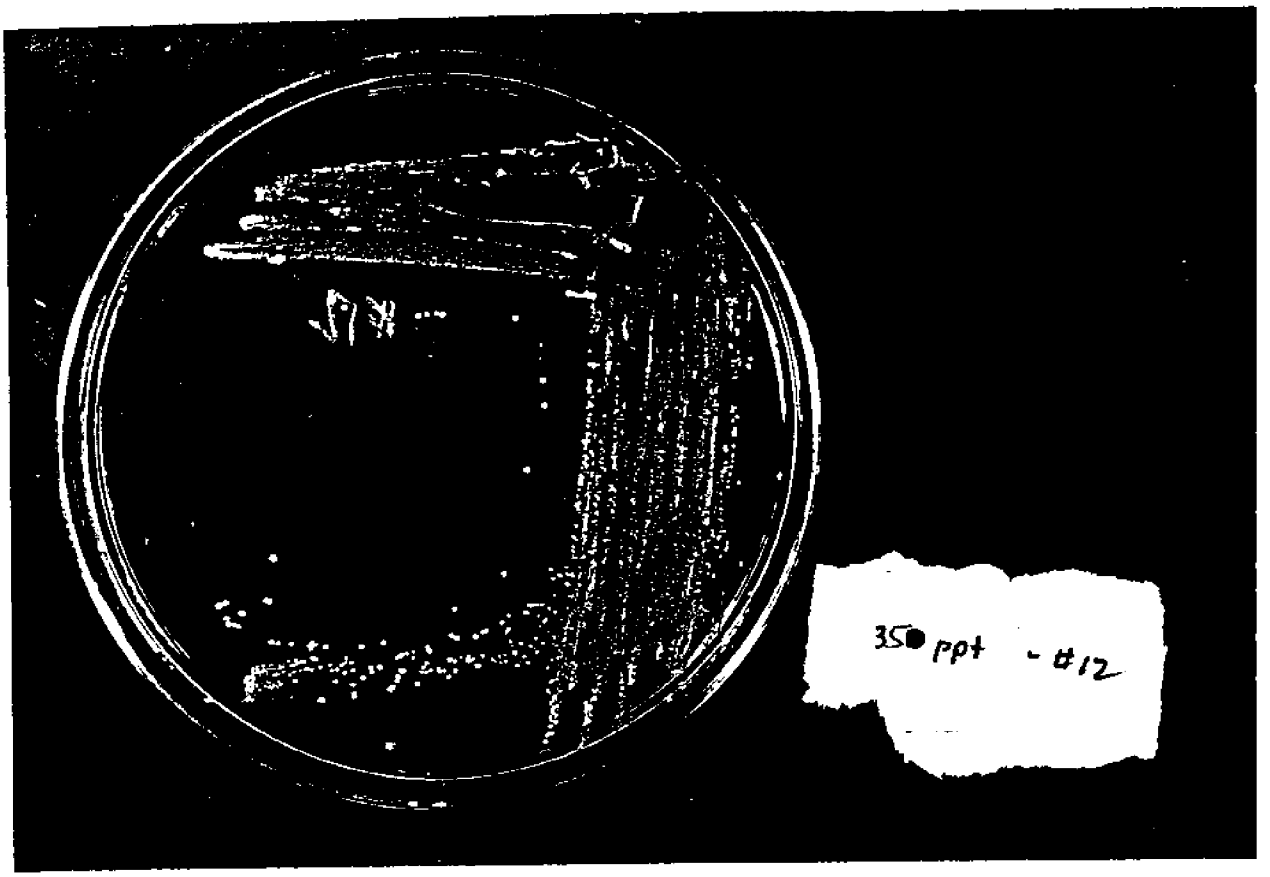

Set 3 - D series 1/100,000 2nd group ($D5_2$) original on 0 ppt salt TSA #4



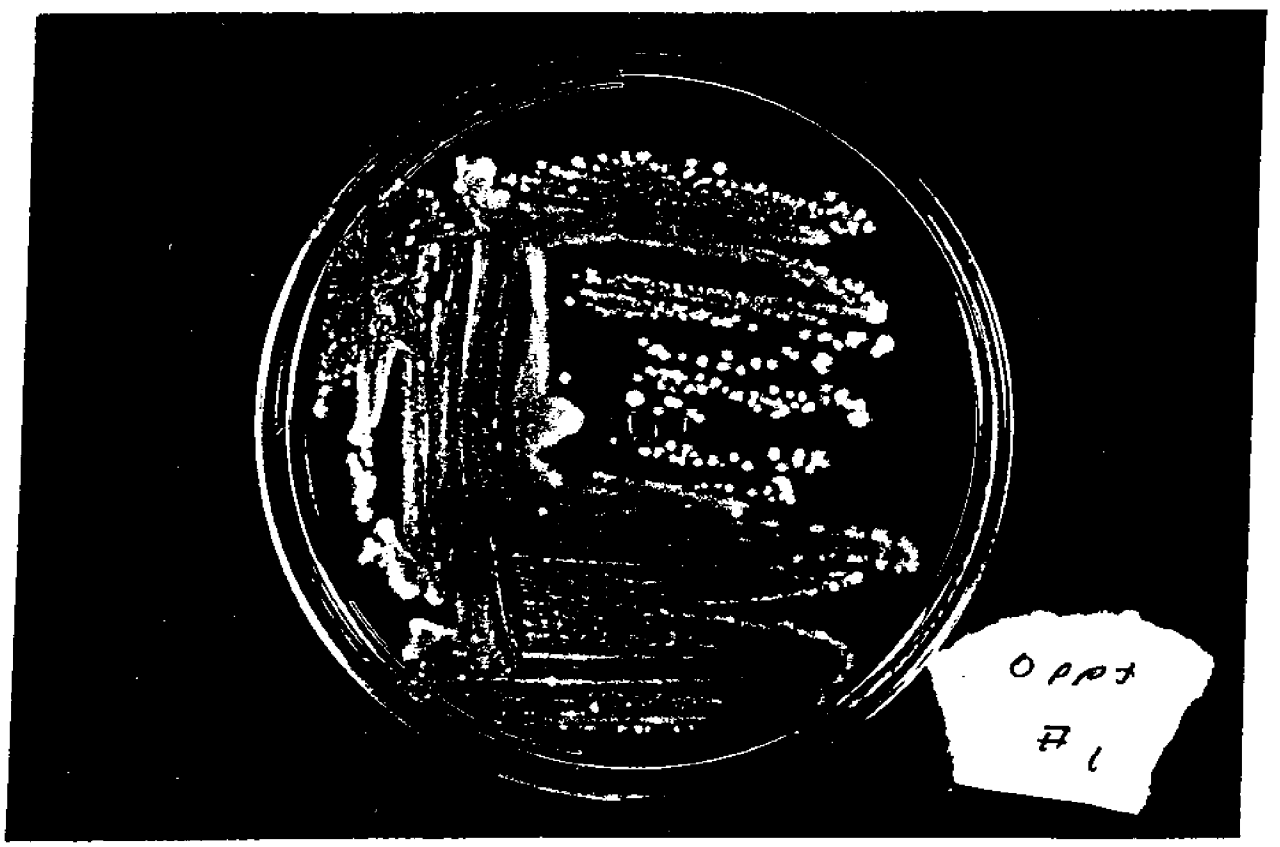

Set 4 - Gradient plate - 35 ppt left → 0 ppt right



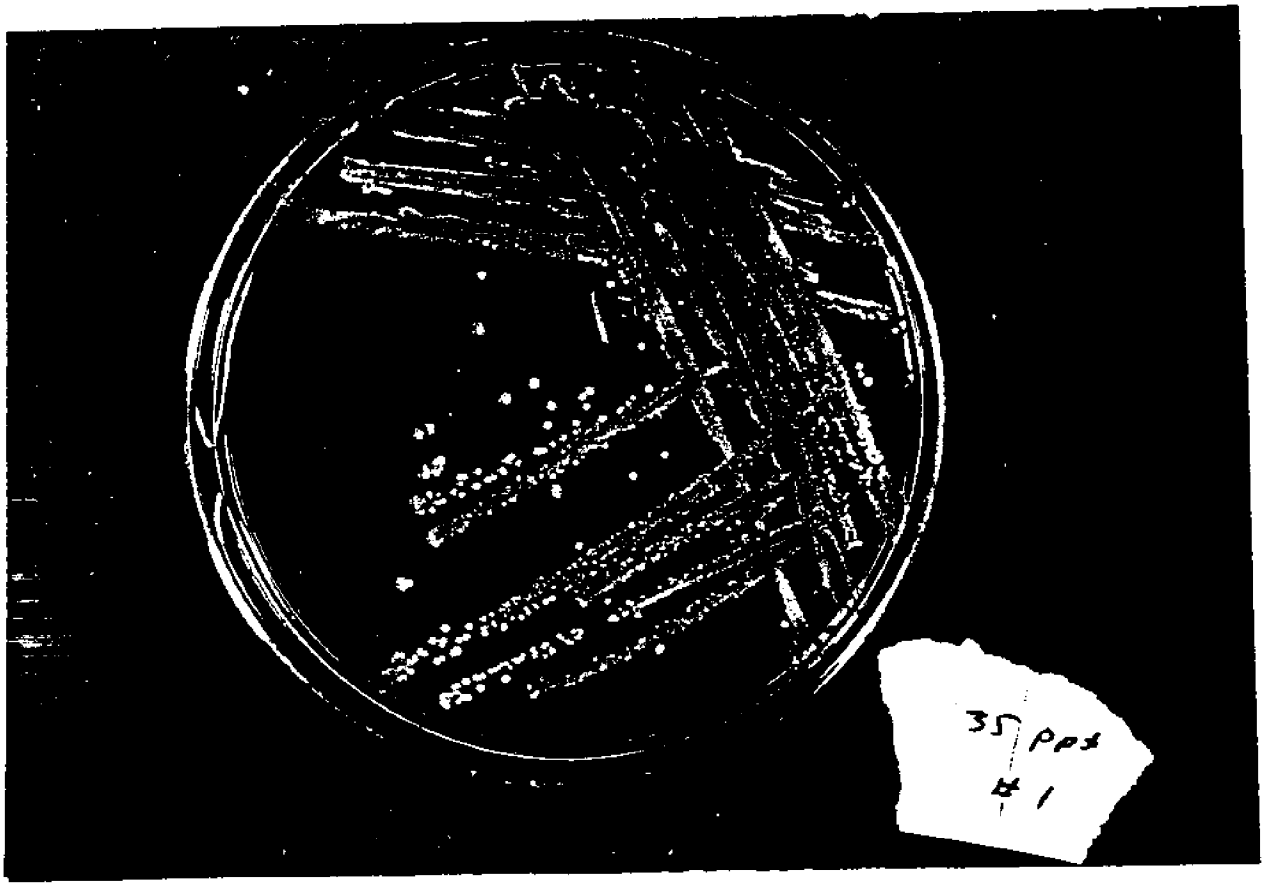

Set 5 - 0 vs 35 ppt colony 12
obligate halophile



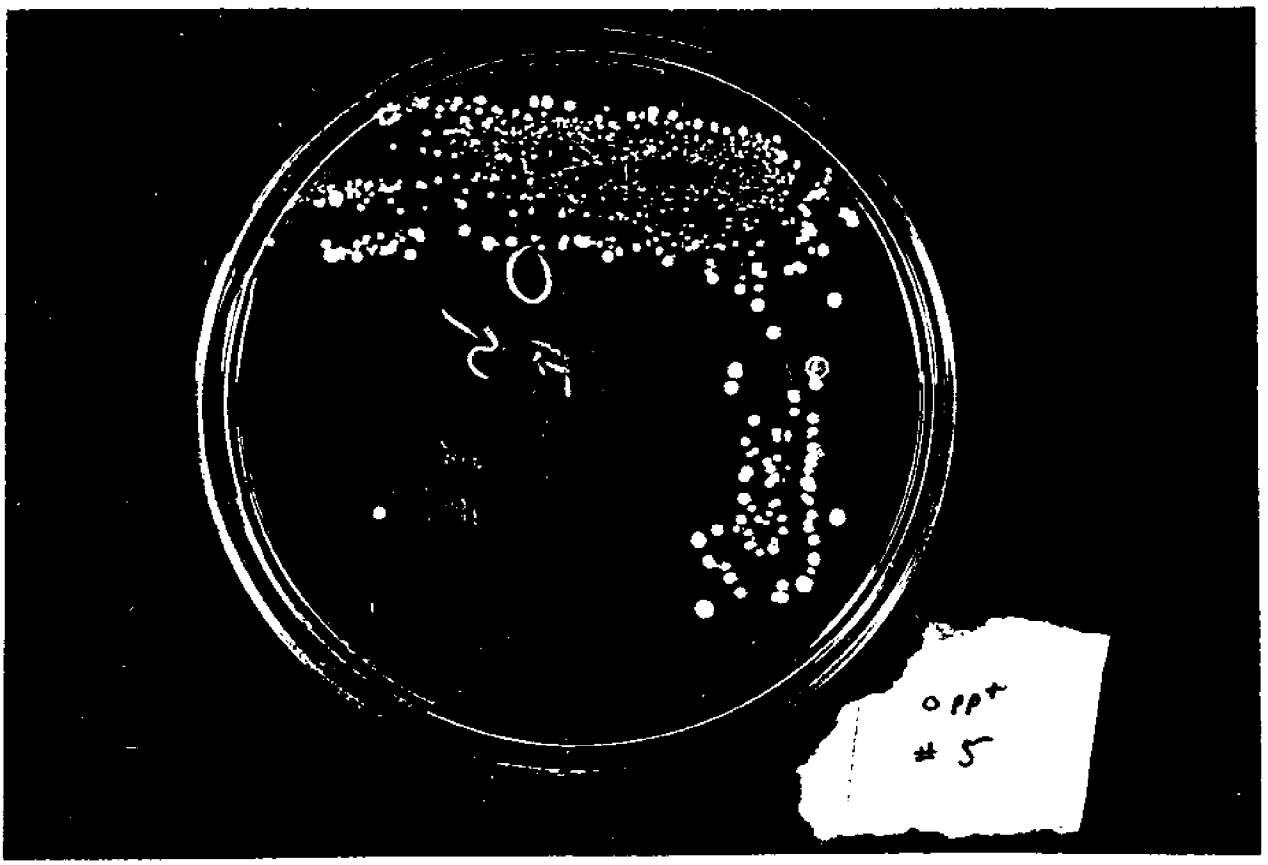

Set 5 - 0 vs 35 ppt - colony 12 obligate halophile #2




Set 6 - 0 vs 35 ppt - colony 1 facultative halophile #1




Set 6 - 0 vs 35 ppt - colony 1 facultative halophile #2




Set 7 - 0 vs 35 ppt - colony 5 #1


Set 7 - 0 vs 35 ppt - colony 5 #2

## Summary Paragraph

This study was designed to determine sea salt requirements of bacteria found in marine sediments of coastal waters. Questions dealt with were 1) Are there any obligate halophiles present and 2) what salt requirements are there for other organisms in the biosystem. Results showed that in marine soil the vast majority of organisms are salt tolerent with relatively few obligate halophiles present. The samples from Bay St. Louis show that due to the varying salinity of the water, all organisms tend to be facultative or salt tolerent organisms. These will grow better in a salt-free environment as shown by the replica plates, but, as there were no obligate halophiles there were no obligate non-halophilic organisms found. This indicates that the biosystem has developed to handle reasonable variations in salt concentration of the bay.

These results can be used to help understand the biosystem of coastal waters, as in Bay St. Louis. As only 3 months were devoted to this study by an undergraduate seeking experience in research, many loose ends were left hanging. Items such as looking further into the techniques of replica and gradient plating and establishing environmental markers other than salt requirements could certainly enhance the usefulness of biomass studies. Such parameters could be applied to organisms already characterized as to their salt tolerance. These characteristics, when routinely monitored, might be a source of information on many food chains in the estuarine environment.