Major Research Project (SR)
Microbiology

F.No. 34-231/2008/(SR), Dated. 26 Dec. 2008.

"Potential research for production of biosurfactant from plant material by using microbial technology."

UNIVERSITY GRANTS COMMISION
NEW DELHI- 110 002

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March-2012

CERTIFICATE

This is to certify that, the Major Research Project entitled, ***"Potential research for production of biosurfactant from plant material by using microbial technology."*** Sanctioned by University Grants Commission, New Delhi vide letter no. **F.No. 34-231/2008/(SR), Dated. 26 Dec. 2008** is a record of bonafide research work carried out by Dr. R.S. Awasthi**,** and this has not formed in whole or in part the basis for the award of any degree or diploma. Work by others has been specifically acknowledged.

Place: Renapur.
Date:

Dr. R.S. Awasthi
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ACKNOWLEDGEMENT

First and foremost my thanks must go to University Grants Commission, New Delhi for the sanctioned of Major Research Project in Microbiology subject with financial assistance, for this great support throughout my research work without which it was impossible to complete this work.

My sincere thanks are due to Honorable Chairman Padmabhushan Dr. Badrinarayanji Barwale and Ex. MLA. Shri. Shivajirao Balvantrao Patil Kavekar for their constant encouragement and facilities rendered to me during my research work.

I am thankful to Principal, Badrinarayan Barwale Mahavidyalaya, Jalna, and Principal Shivaji Mahavidyalaya, Renapur for granting me the permission to carryout my research work in the institution.

I want to record my sincere thanks to Dr. B.S. Nafade, Associate Professor, Dr. S.R. Kagane, Dr. Swati Puri and Mr. Azahar Kazi for their valuable suggestions and scientific support during the entire research work.

I also would like to thank Mr. K. N. Kumbhar and Mr. A.P. Bhande for their cooperation in all respect through out the research work.

I am thankful to the NCL, Pune University and Agricultural Fertilizer testing laboratory, Aurangabad, for their instrumental help during the work.

The libraries play pivotal role in research study. I have conducted my reference work at Jaykar library of Pune University, College of Agriculture Pune, Agarkar research Research Institute Pune, Marathwada Agricultural University Parbhani, Swami Ramanand Teerth Marathwada University Nanded, Dr. Babasaheb Ambedkar Marathwada Univerrsity, Aurangabad. I am cordially thankful to the librarian and other library staff of these institutes for their kind cooperation during my visits.

I am extremely thankful to Mr. Dinesh Shinde for beautification, computerization and presentation of the data in the Project Report.

My sincere thanks are to one and all.

Dr. R.S. Awasthi,
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CHAPTER-I

INTRODUCTION

1.1 INTRODUCTION

Biosurfactants are surface active compounds released by microorganisms. They are biodegradable, nontoxic and ecofriendly materials. Biosurfactants are also described as amphiphilic biological compounds produced extracellulraly or as a part of cell membrane by variety of yeasts, bacteria and filamentous fungi (Chen *et al.* 2007, Mata Sandoval *et al.*, 1999, 2000)

These microorganisms produce biosurfactants using various substrates like sugars, oils and wastes. However carbohydrates and vegetable oils are among the, most widely used substrates for biosurfactant production by *Pseudomonas aeruginosa* strains (Rahman *et al,* 2002a, b, 2003, Raza *et al*, 2007)

The amphiphiles that forms micelles can be potentially used for surface chemical works are termed as surface active agents or surfactants (Pattanathu K.S.M Rahman and Edward Gakps, UK, 2008).

Several biosurfactant, extracellularly produced by microorganism's exhibits properties of emulsifier and their emulsification activity is found to be superior over chemically synthesized emulsifiers (Desai and Banat, 1997).

Biosurfactants have becomes recently an important product of biotechnology for industrial and medical applications. The reason for their popularity, as a high value microbial products, is preliminary in their specific action, low toxicity, relative ease of preparation and wide spread applicability. These surface active agents can be used as emulsifiers, de-emulsifiers wetting agents, spreading agents, foaming agents, functional food ingredients and detergents in various industrial sectors such as petroleum and petrochemicals, organic chemicals, food and beverages, cosmetics, and pharmaceuticals, mining and metallurgy, agrochemicals and fertilizers, environmental control and management and many other industries.

Thus surfactants are used by many industries and one could easily say that there is almost no modern industrial operation where properties of surface and surface active agents are not exploited (N.Kosarik,1992).

These compounds used in industries however produced by microorganisms and have been paid increasing attention to replace synthetic surfactants.

Nowadays, use of biosurfactants has been limited due to the high production cost. Nevertheless, biosurfactants can be produced with high yield by some microorganisms, especially *pseudomonas spp*. Such microorganisms can use various renewable resources, especially agroindustiral wastes, as a potential carbon sources. This leads to great possibility for economical biosurfactant production and reduced pollution caused by those wastes (Suppasil Maneerat, 2005). The use and potential application of biosurfactants in medical field has increased during the past decade. Their antibacterial, antifungal and antiviral activities make them relevant molecules for their applications in combating many diseases and as therapeutic agents. In addition their role as anti-adhesive agents against several pathogen indicates their utility as suitable anti adhesive coating agents for medical insertional materials leading to a reduction in a large number of hospital infections without the use of synthetic drugs and chemicals. (Rodriguez et al, 2006).

One of another such approach of biosurfactant production by alkaliphic bacteria as extremophiles can be used as a source for novel enzymes. (Proteins) (Bertus van den Burg, 2003.)

The term “Aikaliphiles” is used for microorganisms that grow optimally or very well at PH value above 9 and between - 10-12 but can not grow or grow only slowly at near neutral pH value Even alkaliphiles are described as there is no any precise definitions of what characteristics and alkaliphilic or alkalitolerent organisms, because several organisms exhibit more than one pH optimum for growth depending on growth conditions, particularly nutrients, metal ions, and temperature.

The discovery of alkaliphiles was fairly recent because only 16 scientific papers were found on Alkaliphiles: some applications of their products for Biotechnology in 1968 (Koki Horikashi, Dec. 1999) an alkaliphilic microorganisms consist of two main physiological groups.

a) Alkaliphiles

b) Haloalkaliphiles

Since rediscovery of alkaliphilic bacteria shows these are unique microorganisms with great potential of microbiology and biotechnological exploitation. The aspects that have received the most attention in recent years include.

a) Extra cellular enzymes and their genetic analysis

b) Mechanism of membrane Transport and pH regulation.

c) The taxonomy of alkaliphilic microorganisms.(Koki Horikoshi1999).

Thus microbial life does not seem to be limited to specific environments. In last few decade it has becomes clear that microbial communities can be found in must diverse conditions including extremes of pH, Temperature, salinity and pressure which are called as extremophiles which produces proteins/ Biocatalysts / Enzymes which are functional at extreme conditions.

At present very minor fraction of microorganisms on earth have been exploited. Novel developments in cultivation and production of extremophiles but also cloning and expression of their genes in heterogenous hosts will increases no of enzyme driven transformations in chemical, food and other industrial applications. (Bertus Van den Burg, 2003,).

These extremophiles have evolved several structural and chemical adaptations, which allow them to grow in extreme environments. The extremozymes of such microbes function in extreme environments have several biotechnological applications (T.satyanarayan Chandralata Aaghukumar and S.Shivaji current science, 2005).

If such microbes are used for biasurfactant production which in turn exhibit properties of emulsifier and are superior over chemically synthesized emulsifiers/ biosurfactant.

Current world wide biosurfactart markets are around $ 9.4 billion per annum and their demand is expected to increase at a rate of 35% towards end of century. (Desai and Banet, 1997)

The yield of biosurfactant production greatly depends not only upon microorganisms but also nutritional requirement of and other environmental conditions. The present research," **"Potential research for production of biosurfactant from plant material by using microbial technology."** is undertaken to test the production capability of microorganisms for biosurfactant from plant material.

1.2 OBJECTIVE:

The objectives of the present study is to develop a cost-effective fermentation process for the production of biosurfactant by using plant material.

1.3 SCOPE:

1. Isolation and screening of biosurfactant producting microorganisms.
2. Characterization and indentification of biosurfactant producing selected microorganisms.
3. Optimization of pH, temperature, nutrient requirement, incubation period, aeration etc for biosurfactant production.
4. Study of effect of plant material as substrate for biosurfactant production.
5. Primary analysis of Biosurfacetant produced by using plant material.
6. Study biosurfactant production on laboratory scale by using fermentor.

CHAPTER- II
REVIEW OF LITERATURE

2.1 INTRODUCTION:-

The term alkaliphiles is used for microorganisms that grow optimally or very well at pH value above 9, often between 10-12, but can not grow or grow only slowly at the neutral PH or near neutral PH value (6.5)

Alkaliphiles can be isolated from normal environments such as garden soil, though the viable count of alkaliphiles are higher in the samples collected from alkaline environments. The cell surface may play's a key role in maintenance of pH value in the range between 7 to 8.5, allowing alkaliphiles to thrive in alkaline environments, although adaptation mechanisms have not yet been clarified, alkaliphiles have made a great impact in industrial applications.

The discovery of alkaliphiles was fairly recent because only 16 scientific papers were found when experimental work on alkaliphilic bacteria started in 1968 by Horikoshi. The use of alkaliphilic microorganisms has long history in Japan since from ancient times indigo has been naturally reduced under alkaline conditions in the presence of sodium carbonate. Indigo from indigo leaves is reduced by particular bacteria that grow under these highly alkaline conditions in the traditional process called indigo fermentation. After 1971, the industrial applications of these microorganisms have also been investigated extensively and some enzymes put to use on an industrial scale.

Alkaliphiles have clearly gained large amount of genetic information by evolutionary process and exhibit an ability in their genes to cope with particular environments. There fore their genes are a potentially valuable sources or information waiting to be explored and exploited by bacteriologists.

There is no generally agreed definition for alkaliphiles because these Microorganisms can survive in differing pH spectra and definitions have been made with respect to specific pH range (Horikashi, 1999 b).

For-examples those microorganisms whose optimal growth rate is observed at least two pH units above neutrality are called true alkaliphiles. And microorganisms whose optimal growth pH is around or below neutrality are called alkalotolerants since they can also survive at pH points more than- 9 (Kroll, 1990).

On the other hand Horikoshi (1999b) has used the term alkaliphile for microorganisms that grow optimally or very well at pH value 9.0, but can not grow or only grow slowly at neutral PH values.

According to Krulwhich (1989) alkaliphiles can be divided in to two groups

1. Alkaline tolerant showing optimal growth at the pH-7.0- 9.5 but incapable of survival at pH-above 9.5.
2. Alkalophiles showing optimal growth at the pH range 10.0 and 12.0.
   - extreme alkalophiles are further divided among themselves (Krulwich-1989)
   - Facultative alkalophiles showing optimal growth pH -10.0 or above but having also ability to grow well at the neutral pH.
   - Obligate alkalophiles showing optimal growth above pH- 10.0 but incapable of growth below pH- 8.5 - 9.0.

Alkaliphiles have also been categorized in to two main physiological groups as,

a) Alkaliphiles- which requires an alkaline pH of 9 or more for their growth and have an optimum pH of about 10.

b) Haloalkaliphiles- which requires both an alkaline pH (>PH-9) and high salinity up to 33% [w/v] NaCl. Alkaliphles have been mainly found in extremely alkaline saline environments Ex. of such environments are Rift valley lakes of East Africa and Western Soda lakes of United states, one more such extreme alkaliphilic natural environment in India is a ‘Lunar Lake’ in Maharashtra State.

Alkaliphilic microorganisms coexist with neutrpohilic microorganisms as well as also found in specific extreme environments in nature.

To isolate alkaliphiles alkaline media must be used. For pH adjustment of the medium sodium carbonate generally used because alkaliphiles usually requires some sodium ions.

Basal media for isolation of alkaliphilic microorganisms requires following ingredients in the amount mentioned as below.

Table No.2.1.1 Ingredients of medium

Ingredient	Amt. (9/lit) in Horikashi-I	Amt. (9/lit) in Horikoshi-II
Glucose	10	0
Soluble starch	0	10
Yeast extract	5	5
Polypeptone	5	5
KH_2PO_4	1	1
$MgSO_4.7H_20$	0.2	0.2
Na_2CO_3	10	10
Agar	20	20

Many different kinds of alkaliphilic microorganism's including bacteria belonging to the genera *Bacillus*, *micrococcus pseudomonas* and streptomyces and eukaryotes such as yeasts and filamentous fungi have been isolated from variety of environments (Koki Horikoshi, 1999).

As microbial life does not seem to be limited to specific environment during few decades it becomes clear that microbial communities can be found in the most diverse conditions not only to the extreme pH but also Temperature, Salinity, Pressure etc. To fulfill increasing industrial demands for biocatalysts that can cope with industrial process conditions, considerable efforts have been devoted to the search for such enzymes. As compare to chemical synthesis, biocatalysts which have lead to more efficient production of substances.

Still now 3000 different enzymes (current opinion 2003, 6:213-218) have been identified and many of these have found their use in to

biotechnological and industrial applications. The present enzyme toolbox is still not sufficient to meet all demands. The major cause for this was the fact many available enzymes do not withstand industrial reaction conditions. As a result of this characterization of microorganisms that are able to thrive in extreme environments have received great deal of attention as such extrremophiles are valuable source of novel proteins/ enzymes.

The extreme conditions are referred to the physical (Ex. Temperature, Pressure, Radiation) and also to geochemical extremes salinity and pH. (Bertus van den Burg, 2003).

Many extreme environments found in nature which are too harsh for normal life to exist. Any environmental condition that can be perceived as beyond the condition normal acceptable range is an extreme condition (current science vol. 89.No.1 10 July 2005) A variety of microbes can grow and survive at these conditions are called as extremophiles. Thus extremophiles are not only tolerating specific extreme conditions but usually requires these for survival and growth. The range of environmental extremes tolerated by microorganisms is much greater than life forms. The limit for growth and reproduction of microbes are -12^0c to $+100^0$c, pH 0 to 13 hydrostatic pressure 1400 atm and salt concentration of saturated brines. (T .satyanarayana, Chandralata Raghukumar and S.Shivaji, 2005).

2.2 PHYSIOLOGY OF ALKALIPHILES

Most alkaliphiles have an optimal pH at around 10 , which is most significant difference from well investigated neutrophilic microorganisms can grow in such an extreme environment. Is there any difference between physiological and structural aspects between alkaliphilic and neutrophilic microorganisms? Internal cytoplasmic pH can be estimated from the optimal pH of intracellular enzymes. For example, α-galactosidase from alkaliphile, *Micrococcus spp.* Strain as below in the table had its optimal catalytic pH at 7.5, suggesting that internal pH is around neutral. Furthermore, the cell-free protein synthesis system from alkaliphiles optimally incorporate amino acids in

to protein at pH at 8.2 to 8.5 ,only 0.5 pH higher than that of the neutrophilic *Bacillus subtilis* .

Table NO 2.2.1 relation between external and internal pH.

Microorganism	External pH	Internal pH
B.alkalophilus	8.0 9.0 10.0 11.0	8.0 7.6 8.6 9.2
B.firmus	7.0 9.0 10.8 11.2 11.4	7.7 8.0 8.3 8.9 9.6
Bacillus strain YN-2000	7.5 8.5 9.5 10.2	8.5 7.9 8.1 8.4
B.halodurans c-125 Intact cells	7.0 7.5 8.0 8.5 9.0 9.5 10.0 10.5	7.3 7.4 7.6 7.8 7.9 8.1 8.2 8.4
Protoplasts	7.0 8.0 8.5 9.0 9.3	7.5 7.9 8.2 8.4 8.6

Another method to estimate pH is to measure in cell the inside and outside distribution of weak bases, which are not actively transported by cells. The internal pH was maintained at around 8.0 despite a high external pH of 8.0-11.0 as shown in above table. Therefore, one of the key features in alkalophily is associated with the cell surface, which discriminates and maintains the intracellular neutral environment separate from the extra cellular alkaline environment.

2.3 CELL WALLS OF ALKALIPHILIC BACTERIA

2.3.1 Acidic polymers

Since the protoplast of alkaliphilic bacteria like *Bacillus* strains looses their stability in alkaline environments, it has been suggested that the cell wall may play a key role in protecting the cell in alkaline environments. Compounds of the cell of several alkaliphilic Bacillus spp .have been investigated in comparison with those of the neutrophilic *B.subtilis* .in addition to peptidoglycan, alkaliphilic *Bacillus spp.* Certain acidic polymers such as galacturonic acid (Anono,R,and K.horikoshi,1983).The negative charge on acidic non peptidoglycan components may give the cell surface its ability to adsorb sodium and hydronium ions and repulse hydroxide ions and as a consequence, may assist cell to grow in alkaline environment.

2.3.2 Peptidoglycan

The peptidoglycans of alkaliphilic *Bacillus spp*. appear to be similar that to of *B.subtilis*. However, their composition was characterized by an excess of hexosamines and amino acid in the cell walls compared to that of the neuterophilic *B.subtilis* .Glucosamine, muramic acid, D and L- alanine, D-glutamic acid, meso-dimino pimetic acid and acetic acid were found in hydrolysates. Although some variation in the amide content among the peptidoglycan from alkaliphilic *Bacillus* strain was found, the variation in pattern was similar to that known in neutrophilic *Bacillus* species.

2.3.3 Na^+ Ions and Membrane Transport

Alkaliphilic microorganisms grow vigorously at pH 9 to 11and requires Na^+ for growth. The presence of sodium ions in the surrounding environment has proved to be essential for effective solute transport through the membranes of alkaliphilic *Bacillus spp*. According to chemiosmotic theory, the proton motive force in the cells is generated by electron transport chain or by excreted H^+ derived from ATP metabolism by ATpase. H^+ is then reincorporated in to the with co transport of various substrates. In Na^+-dependent transport systems, the H^+ is exchanged by Na^+ by Na^+/H^+ antiporter systems, thus generating a sodium motive force ,which drives substrates accompanied by

Na^+ in to the cells. The incorporation of a test substrate Alfa aminobutyrates, increased two fold as the external pH shifted from 7 to 9,and presence of sodium ions significantly enhanced the incorporation ; 0.2 N NaCl produced an optimum incorporation rate that was 20 times the rate observed in the presence of NaCl. Other cations including K^+, Li^+, Cs^+ and Rb^+, showed no effect, nor did their counteranions.

2.4 Mechanisms of Cytoplasmic pH Regulation

The cells have two barriers to reduce pH values from 10.5 to 8.

Cell walls containing acidic polymers functions as a negatively charged matrix and may reduce the pH value at the cell surface .The surface of plasma membrane must presumably be kept below pH 9, because plasma membrane is very unstable at alkaline pH values(8.5 to 9)much below the pH optimum for growth.

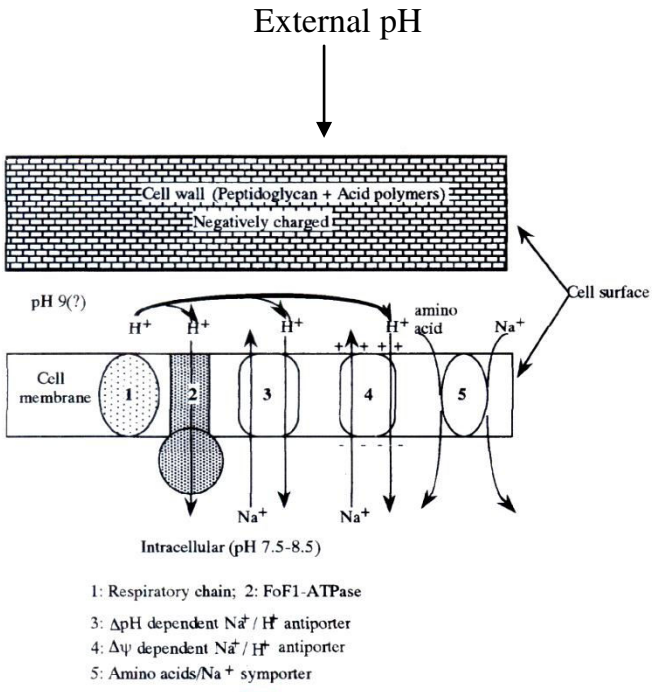


Fig: 2.4.1 Cytoplasmic pH Regulation Scheme in alkaliphilic Bacteria.

Plasma membranes may also maintain pH homeostasis by using the Na^+/K^+ antiporter system, the $K^{+/}$ H^+ antiporter and ATpase –driven H^+ expulsion. Recent studies on the critical antiporters in several laboratories have

begun to clarify the number of characteristics of the porters that support active mechanism of pH homeostasis.

Krulwich *et al* .have focused their studies on facultative alkaliphilic *B.firmus* OF4, which is routinely grown on malate containing medium at either pH 7.5 or 10.5. current work is directed towards clarification of the characteristics and energetic of membrane associated proteins that must catalyzes inward proton movements one such protein is the Na^+/H^+ antiporter, which enable the cells to adopt to sudden upward shift in PH and to maintain a cytoplasmic pH 2 to 2.3 units below the external pH in the most alkaline range of pH for growth. Another is proton translocation ATP synthesis that catalyzes production of ATP under conditions in which the external proton concentration and the bulk chemiosmotic driving force are low. Three gene loci that are candidates for Na^+/ H^+ antiporter, carrying genes with roles in Na^+ - dependent pH homeostasis, have been identified. All of them have homologous in *B.subtilis*, in which pH homeostasis can be carried out with either K^+ or. Na^+ The physiological importance of one of the *B.furmus*, OF4 loci, nhaC, has been studied by targeted gene disruption, and the same approach is being extended to the others. The atp genes which encode the F_1F_0-ATP synthesis of the alkaliphile have interesting motifs in areas of putative importance for proton translocation. As an initial step in studies that will probe the importance and possible role of these motifs, the entire atp operon from *B.firmus* OF4 has been cloned and functionally expressed in an *E.coli* mutant with its atp genes completely deleted. The transformation does not exhibit growth on succinate but shows reproducible, modest increase in the aerobic growth yield on glucose as will as membrane ATPase activity that exhibit characteristics of the alkaliphile enzymes. As a result of these mechanisms, the membrane proteins plays an important role in keeping the intracellular pH values in the range between7.0 to8.5. (Koki Horikosha, 1999).

The structural components in cell walls of three mutants of a facultative alkaliphile, *Bacillus lentus* C-125, defective in certain cell-wall components were characterized in detail. The cell walls of the wild-type C-125 were thick

and increased in thickness when grown at high pH. Electron microscopy showed that triple layers developed when the bacteria were grown in an alkaline environment. In contrast, cell walls of teichuronopeptide (TUP) defective mutants consisted of a single layered. For both the wile-type and mutants, the cell-wall concentrations of the acidic structural polymers teichournic acid and TUP increased with respect to peptidoglycan as culture pH increased. For all four strains, the anion content of their cell walls was the greatest at high pH. The cell-wall density of the negatively charged compounds (uronic acids plus L- glutamic acid) was calculated as about 3 and 9 equivalents (I cell wall region) for C-125 cells grown at pH 7 and 10, respectively. At high pH, the specific growth rates of the two TUP –defective mutants were much lower than those of the wild-type. It is concluded that increased levels of acidic polymers in the cell walls of alkaliphilic bacteria may be a necessary adaptation for growth at elevated pH.

The facultative alkaliphile bacillus lentus C-125 grows between pH 6/8 and 10/8. This organism used to be called bacillus sp C-125 (Aono & Horikoshi, 1983): however, recently this organism was shown to be related to *B. leutus* (Aono 1995). It has been reported that A-dependent Na^+/H^+ antiporter on the cell membrane helps to maintain the intracellular pH around neutrality (Kitada *et al.*1994). The bacterial cell wall also appers to be important for the development and maintenance of alkaliphily. Protoplasts prepared from strain C-125 are unstable at alkaline pH (Aono et al, 1992) and regenerate at neutral pH but not at alkaline pH (Aono *et al.*, 1993). The cell wall of alkaliphiles possibly acts as an obstacle to the high extra cellular concentrations of hydroxide ions and as a reservoir of hydrogen ions generated intracellularly by respiration.

The cell walls of *B.leutus* C-125 contain peptidoglycan, teichuronic acid (TUA) and teichuronopetide (TUP). The peptidoglycan is and Alz type that is identical to that in the cell walls of the neutrophilic *Bacillus subtilis* (Aono *et al.*, 1984). The TUA consists of galacturonic acid, glucuronic acid and N-acetly-D-2 amino-2, 6-dideoxyglactose (fucosamin) in a molar ratio of 1:1:1

(Aono & Uramoro, 1986). The TUP is a polymer in which polygluramic acid binds covalently to polyglucuronic acid (PGU) and has a 1:5 molar ratio of glucuronic acid to L- glutamic acid (Aono 1985, 1987, 1989). The quantities of acidic polymers in the cell walls of strain C-125 increase when cells are grown at alkaline pH (Aono & Horikoshi, 1983; Anon, 1985). Mutants, defective in the acidic polymers grow poorly on an alkaline solid medium (Aono & Ohtani, 1990; Ito, *et al.* 1994)

These studies suggested that the acidic polymers in the cell walls might be important for bacterial growth in an alkaline environment. However, this conclusion was drawn from experiments with cells from cultures grown an initial pH values of 7.0 or 10.0 but lacking pH control during growth. Moreover, there have been few detailed. A measurement of the composition of the cell walls of alkaliphiles and little is known about the effect of culture pH on cell wall morphology. Thus cell wall defective mutants grown under pH control. And detailed pH-dependent alteration of the chemical composition of cell wall of the C-125 wild type and the mutants, the pH dependent specific growth rates of the strains, and the ultra structure of cell wall of the strains grown at several pH values (Rikizo Aono, masahiro Ito, Keith N.Joblin and Koki Horikoshi, 1995).

2.5 PYSICAL MAPS OF CHROMOSOMAL DNAs OF ALKALIPILIC BACILLUS STRAINS

How alkaliphiles adapt to their alkaline environments is one of the most interesting and challenging topics that might be clarified by genome analysis. To physical maps for alkaliphilic bacillus strains have been published: those for *B. firmus* OF4 and *B. halodurans* C-125.A physical of *B.firmus* OF4 is consistent with a circular chromosome of approximately 4 Mb with an extra chromosomal element of 110 kb (Gascoyne, D.J., J., A Connor, and T.ABull.1991). Although analysis is still in progress, several open reading frames for Na^+ /H^+ antiporters that may play roles in pH homeostasis have been detected. A physical map of the chromosome of *B. halodurans* C-125 has been constructed to facilitate whole genome analysis (Takami, H.,Y., Takaki,

Nakasone, C.Hirama, AInoue and K. Horikoshi, 1999; Takami, H.K., Nakasone, C .Hirama, Y. Takai N., Masui, F, Fuji Y. Nakamura and A, Inous, 1999). In this strain, the genome size is 4.25 Mb. Many open reading frames showing significant similarities to those of *B. Subtilis* have been positioned on the physical map. As shown in Fig. below, the oriC regions of the C-125 chromosome have been identified by Southern blot analysis with a DNA probe containing the gyr B .region(Takami, H.,Y., Takaki, Nakasone, C.Hirama, AInoue and K.Horikoshi, 1999; Takami, H.K., Nakasone,C. Hirama. Y. Takai, N. Masui, F, Fuji, Y. Nakamura and A, Inous, 1999). Whole-genome analysis will inevitably give us useful information about adaptation to alkaline environments (Koki Horikoshi, 1999).

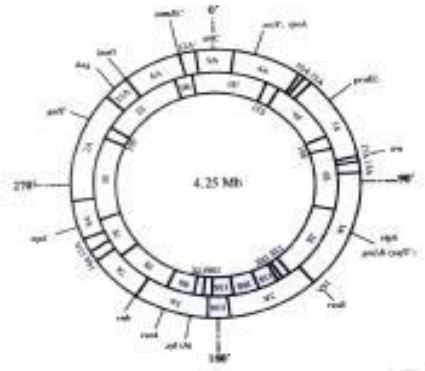


Fig: 2.5.1 genetic Map of *B.halodurans*

Alkaliphiles maintain a neutral or slightly alkaline cytoplasm. The intracellular pH regulation is dependant on presence of sodium, which is exchanged from cytoplasm in to medium by H^+/Na^+ antiporters. Electrogenic proton extrusion is mediated by respiratory chain activity and protons are transported back in to the cell via antiporters, which are efficient at transporting H^+ in to the cell at the expense of Na^+ export from it. Besides controlling protons, Na^+ dependent pH homeostasis requires reentry of Na^+ in to the cell. Na^+- coupled solute symporter and Na^+-driven flagella rotation ensure a net sodium balance (Horikoshi .K, 1998, Yayabos A.A., Diez, A.S. and Van BoxteL R, 1991). The combined action of antiporters coupled with respiration

provides the cell with a means of controlling its internal pH, while maintains its adequate Na^+ levels through symport and flagella rotation. The peptidoglycan layer of alkaliphiles has a higher cross linking rate at higher pH values. This may provide shielding effect by "tightening' the cell wall. Sodium gradient is used to energize solute transport and flagella movement, but not for ATP synthesis. Sodium dependent ATP syntheses have not been identified in alkaliphililes. Further, ATP synthesis in alkaliphilic *bacillus* species have been demonstrated to be exclusively proton translocating.

Major impetus that have driven extensive and intensive research efforts on extremophiles during last decades is the potential biotechnological applications associated with the microbes and their products. Examples of the extremozymes that are now commercially used include alkaline proteases in detergents. It is a huge market, with 30% of total worldwide. Production for detergents in 1994, the total market for alkaline proteases in Japan was around 15,000 million yens.

Table No 2.5.1.1 Potential applications of extremophiles (alkaliphiles) in biotechnology.

Source Alkaliphile	**Use**
Protease, Cellulose, Xylanase, Lipase and pullulaneases	Detergents.
Proteases	Gelatin removal on x-ray film.
Elastases, keritinases Cyclodestrins	Hide deharing food stuffs, chemical is and pharmaceuticals.
Sylenases/proteases	Fine papers, waste treatement and degumming.
Alkaliphilic halophiles	Oil recovery

various microorganisms	Antibiotics.

The extensive and intensive efforts world over in understanding the diversity of extremophilic microbes have indicated that what we know today is just the tip of iceberg. Many more novel and useful extermophilic microorganisms are expected to be discovered in the near future. Only sporadic attempt have been made to isolate extremophiles from extreme Indian environments. Extensive finding and concerted and collaborative efforts are needed for understanding the diversity of these microbes from extreme environments of Indian subcontinent and their exploitation (T .satyanarayana, Chandralata Raghukumar and S.Shivaji, 2005).

If such microbe's extremophiles (alkaliphiles) are used in production of surface active agents called surfactants is one of another commercial application of alkaliphiles for industrial production. (Koki Horikoshi, 1999)

Enzymes from microorganisms that can survive under extreme pH could be particularly useful for applications under highly acidic or highly alkaline reaction conditions, for example in the production of detergents. However one of the striking properties of acidophilic and alkaliphilic microorganisms is their ability to maintain a neutral pH internally and so the intracellular enzymes from these microorganisms do not need to be adapted to extreme growth conditions. However, this does not account for extra cellular proteins, which have to function in low or high pH environments in the case of acidophiles and alkaliphiles, respectively.

Proteases, amylases, lipases and other enzymes that are resistant to and active at high pH and high chelator concentrations of modern detergents are desirable. This has prompted the screening of alkaliphilic bacteria and Archaea for their ability to produce such enzymes. By these means, several useful enzymes have already been identified and obtained. Combinations of homology- based PCR and activity screening have been applied to screen for and detect alkaline proteases in a collection of thermo acidophilic archaeal and

bacterial strains isolated from hot environments (Kocabiyik S and Erdem B., 2002). In an alternative approach, alkaliphilic bacilli that could grow at pH 9 were used as a source for oxidation-resistant alkaline proteases .

Polymer-hydrolysis related processes have also initiated the search for biocatalysts from acidophiles. Several enzymes used for starch-hydrolysis (e.g. amylases, pullulanases, glucoamylases and glucosidases that are active at low pH have been isolated. (Bertus van den Burg, 2003).

The initial motivation for studying alkaline lipase was its application to detergents; many alkaline lipases were significantly inhibited in the presence of either alkylbenzene sulfate or dodecyl benzene sulfonate. (Koki Horikoshi, 1999)

Watanable *et al.* (1977) conducted an extensive screening for alkaline lipase producing microorganisms from soil an water samples. Two bacterial strains were selected as a potent producer of alkaline lipase. These are identified as *pseudomonas nitroreducens* novo themotolerent and *P.frogi*. The optimum pH of the two lipase was 9.5. Both enzymes were inhibited by bile salts such as sodium chloride, sodium deoxycholate and sodium taurocholate at 0.25%. However further work has not been reported (Koki Horikoshi, 1999).

The alkaliphiles are unique microorganisms, with great potential for microbiology and biotechnology; alkaliphilic enzymes should find additional use in various fields of industry, such as chiral- molecule synthesis. Biological wood pulping and more production of sophisticated enzyme detergents (Koki Horikoshi, 1999 p.no.746).

2.6 SURFACTANTS

2.6.1 Definition

Surfactants are amphipathic molecules with both Hydrophilic and hydrophobic (generally Hydrocarbon) moieties that partition preferentially at the interface between fluid phages with different degrees of polarity and hydrogen bonding such as oil/water or air/water interface (Desia and Banat, 1997).

These properties renders surfactants capable of reducing surface and interfacial tension and forming micro emulsion where hydrocarbons can solubilize in water or where water can solubilize in hydrocarbons. These characteristics confer excellent detergency, emulsifying, foaming and dispersing traits, which makes surfactants some of the most versatile process chemicals (Greek, B.F., 1991,1990).

Or

The term' surfactant' is a blend of surface acting agents. Which are usually organic compounds that are usually amphipilic, meaning they contain both hydrophobic groups (their tails) hydrophilic groups (their 'heads') therefore are soluble in both organic solvents and water? The term surfactant was coined by Antara products 1950.

Or

Surfactants are hydrophilic molecules with both hydrophilic and hydrophobic domen. The former can be nonionic, positively or negatively charged or amphoteric, but later is usually hydrocarbon (Geogioue et al, 1992, Desia et al, 1994). Common nonionic surfactant include ethoxylate, ethylene and propylene oxide copolymers sorbitan esters, while fatty acids, ester suifonates sulphates (anionic) and quaternary ammonium salts (cationic) are some examples of commonly available surfactants. Since both the hydrophilic and hydrophobic groups reside with same molecule, surfactants tend to partition preferentially at the interface between fluid phases with a different degree of polarity and hydrogen bonding such as oil/water or air/ water interfaces (Desia and Banat, 1997) Unique properties of surfactant molecules were driven by reduction of interfacial energy (interfacial tension) and surface tension through formation of an ordered molecular film at the interface (Tan H.M-200). These properties allow them to be used extensively in industrial application involving emulsification, foaming, detergence, wetting and phase dispersion or solubilization (Greak. B.F. 1990, 1991).

2.6.2 A] CHEMICAL SURFACTANTS

Chemically synthesized surfactants are classified according to their polar grouping surfactants reduces surface tension of water by adsorbing at the liquid-gas interface. They also reduce the interfacial tension between oil and water by adsorbing at the liquid- liquid interface. Many surfactants can also assemble in the bulk solution in to aggregates. Examples of such aggregates are vesicles and micelles. The concentration at which surfactant begins to form micelles is known as critical micelle concentration (CMC) when micelles form in water, their tail forms a core that can encapsulated an oil droplet, and their (ionic/polar) heads form an outers shell that maintains favorable contact with water. When surfactants assemble in oil, the aggregate is referred to as a reverse micelle. In a reverse micelle, the heads are in the core and the tail maintains favorable contact with oil. Surfactants are also often classified in to four primary groups;

A) Anionic

B) Cationic

C) Non-ionic

D) Zwitterionic (dual charge)

Thermodynamics of surfactant systems are of great importance, theoretically and practically. This is because surfactant systems represent ordered and disordered state of matter. Surfactant solutions may contain an ordered phase (micelles) and a disordered phase (Free surfactant molecules and/ or ions in the solution)

Ordinary washing up (dishwashing) detergents, for ex, will promote water penetration in soil, but the effect would only last a few days. However many standard Laundry detergents powers contain levels of chemicals such as sodium and boron, which can be damaging to plants and should not be applied to soil.

2.6.3 B] BISOURFACTANTS

Biosurfactants are microbial surface active agents (N.kosaoic, 2001)

Or

Biosurfactants are amphipilic biological compounds produced extracellularly or as a part of the cell membrane by variety of yeasts, bacteria and filamentous fungi (Chen *et al*, 2007, Mata Sandoval *et, al*. 1999, 2000) form various substance including sugars, oils and wastes. However hydrocarbons and vegetable oils are among the most widely used substrates for research on biosurfactant production by *pseudomonas aeuroginosq* strains (Rahaman *et, al*. 2002, a,b, 2003, Raza *et al.,* 2007)The amphipiles that form micelles can be potentially used for surface chemical works, are called as surface active agents or surfactants. Soap and detergents can be described as having similar characteristics as surfactants.

All surfactants have two ends namely, hydrocarbon parts which is less soluble in water (hydrophobic end). The hydrophobic part of the molecule is long chain fatty acids, hydroxyl fatty acids, hydroxyl fatty acids or alky/- B-hydroxyl fatty acids. The water soluble end (hydrophilic) can be carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol. Additionally, the hydrophobic moiety is usually C-8 to C-22 alkyl chain or alkyllaryl than may be linear or branched (Van Grinket, 1989) (Pattanathu K.S.M.Rahmanand Edward Gakpe, 2008)

2.6.4 Classification of Biosurfactants

A large No. of microorganisms produces potent surface active agents called biosurfactants, which vary with their chemical properties and molecular size. While the low molecular weight surfactants are often glycolipids, the high molecular weight surfactants are generally either polyaninic or heteropolysaccharides containing co-valently linked hydrophobic side chains or complexes containing both poly saccharidse and proteins. The yield of biosurfactant greatly depends on nutritional requirement of growing organism.

Biosurfactants are classified according to the to the nature of their polar grouping, biosurfactants are categorized mainly by their chemical composition and their microbial origin. In general, their structure includes a hydrophilic moiety consisting of amino acids or peptides anions or cations;

mono-, di-, or polysaccharides; and a hydrophobic moiety consisting of unsaturated, saturated, of fatty acids, accordingly the major classes of bio-surfactants include Glycolipids, Lipopetides and Lipoproteins, Phospholipids and Fatty acids, Polymeric surfactants, and Particulate surfactants.

Although there are a number of reports on the synthesis of bio-surfactants by hydrocarbon-degrading microorganisms, some bio-surfactants have been reported to produced on water-soluble compounds such as glucose, sucrose, glycerol or ethanol (CooperD.G and B.G Goldenberg,1987 Guerra Santos L.H.,O.Kappli and fiechter 1986 Hommel,R.K., LWeber, U. Himelreich O., Rilkeand, H. P., Kleber, 1984, Palejwala S and J.D. Desai 1989, Passeri A, 1992). The bio-surfactant-producing microbes are distributed among a wide variety of genera. The major types of bio-surfactants, with their properties and microbial species of origin, are listed below in the table and are described briefly in the following section.

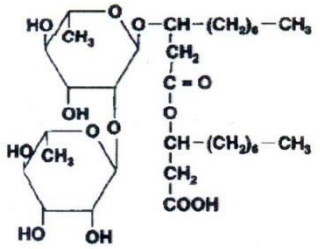
2.6.4.1 Glycolipids

Glycolipids are carbohydrates in combination with long-chain aliphatic acids or bydroxyaliphatic acids. Among the glycolipids, the best known are Rhamnolipids, Trehalolipids, and Sophorolipids.

2.6.4.2 Rhamnolipids

In this type one or two molecules of rhamnose are linked to one or two molecules of β-hydroxydecanoic acid are the best-studied ghycolipids. Production of rhamnose-containing glycolipids. Production of rhamnose-containing ghycolipids was first described in *Pseudomonas aeruginosa* by Jarvis and Johnson (1949). L- Rhamnosyl-L-rhymosil-L-ghamnosly1-Bhydroxydecanoate and L-rhamnosyl-B-hydroxydecanoate, referred to as rhamnolipid 1 and 2, respectively, are the principal glycolipids produced by *P.aeruginosa* (Edward J.R.and J.A.Hayashi, 1965, HisatsukaK.T.Nakahara, N.Sanoand K Yamada 1971, Ito and T Suzuki 1972, Itoh.S.and H .Honda, F.TomitaandTsuzuki 1971) . The formation of rhamnolipid types 3 and 4 containing one B-hydroxydecanoic acid with one and two rhamnose units, respectively (Syldatk, C., S Lang and F Wagner 1985), methyl ester

derivatives of rhamnolipids 1 and 2 (Hirayama,T and I Kato 1982), and rhamnolipids with alternative fatty acid chains (Lang S and F .Wagner1987, Parra,J.L .,Guinea M.A.Manresa,Mrobert M.E.Mercade F Comells and M P. Bosch1989 ,Rendell, N. B.,G ,W.Taylor ,SomerVIlle,H.Todd,R.Wilson and J.Cole1990) has also been reported. Rhamnolipids from *Prseudomonas spp* have been demonstrated to lower the interfacial tension against N-hexadecane to 1 mN/nm and the surface tension to 25 to 30 mN/m (Cuerra –Santos,L.H.,O .Kappli and Flecther; Lang S and F .Wagner1987 , Parra,J.L .,Guinea M.A.Manresa,Mrobert M.E.Mercade F Comells and M P. Bosch1989) . They also emulsify alkanes and stimulate the growth of *P. aenuginosa* on hexadecane. (HisatsukaK.T.Nakahara, N.Sanoand K Yamada 1971. (Itoh and Suzuki1972) isolated two mutants of *P.aeruginosa*, PU-1 and PU-2 which grew poorly on alkanes due to their inability to produce rhamnolipids. These mutants grew normally when the growth medium was supplemented with rhamnolipid.

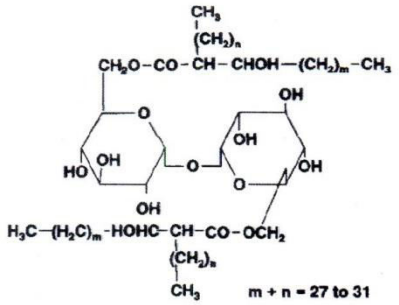


(A)
RHAMNOLIPID

2.6.4.3 Trehalolipids

Another group of glyocolipids are the trehalolipids, the serpentine group seen in many members of the genus *Mycobactertium* is due to the the presence of trehalose esters on the cell surface (Asselineau and asselineau, 1978). Disaccharide trehalose linked at C-6 and C-6' to mycolic acid is associated with most species of *mycobacterium*, *Norcardia* and *Corynebacterium.* Mycolic acids are long-chain α-branched-β-hydroxy fatty acids. Trehalolipids from different, organisms differ in the size and structure of mycolic acid, the

number of carbon atoms and the degree of unsaturation (Desai and Banat, 1997). Trehalaose lipids form *Rhodococcus erythropolis* and *Arthrobacter sp.* Were found to lower the surface and interfacial tensions in culture broth from 25-40 and 1-5 mNm^{-1} , respectively (Li *et al.*,1984).



(B)

TREHALOLIPID

2.6.4.4 Lipoproteins and lipopeptides

Lipopepetides called surfactin are produced by *Bacillus sp.* containing seven amino acids bonded to a carboxyl and hydroxyl groups of a 14-carbon acid. Surfactin just as any other biosurfactant reduces surface tension form 72-27 mNm^{-1} ,with concentrations as low as 0.005%, making surfactin one of the most powerful biosurfactants (Kakinum a *et al.* 1969). The cyclic lipopeptide surfactin produced by *bacillus subtilis* ATCC 21332 is an example of one to the most powerful biosurfactants. Another important characteristic of surfactin is its ability to lyse mammalian erythrocytes and to from spheroplasts (Bemheimer and avigad, 1970). This property is been sued to detect surfactin production through on blood agar.

2.6.4.5 Fatty acids

Fatty acids produced from alkanes as a result of microbial oxidations have been considered as surfactants (Rehn and Reiff, 1981). In addition to the straight-chain acids, micro-organisms produce complex fatty acids containing OH groups and alkyl branches. Examples of such complex acids include Corynomucolic acids that are also surfactants (Kretschner *et al.*, 1982). The hydrophilic or lipophilic balance of fatty acids is clearly related to the length of the hydrocarbon chain. For lowering surface and interfacial tensions, the most

active saturated fatty acids are in the range of C12-C14 (Rosenberg and Ron, 1999)

2.6.4.6 Phospholipids

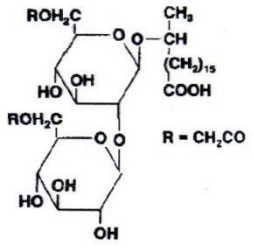
These are known to from major components of microbial membranes. When certain hydrocarbon-degrading bacteria or yeast are grown on alkane substrates, the level of phospholid increases greatly. For instance, using hexadecane-grown *Acinetobacter sp.* HO1-N, Phospholipids (mainly phosphatidylethanolamine) rich vesicles were produced (Kaeppeli and Finnerty, 1979) Phospholipids have been quantitatively produced from *Thiobacillus thiooxidans* that are responsible for wetting elemental sulphur necessary for growth (Beeba and Umbriet, 1971). Phosphatidylethanolamine produced by *Rhodococcus erythroplis* grown on n-alkane resulted in the lowering of interfacial tension between water and hexadecane to less than 1 $mNm^{-1,}$ and CMC of 30 mg L^{-1}, (Kretschneretal.1982).

2.6.4.7 Polymeric Biosurfactants

Emulsion, liposan, Mannprotein and polysaccharide-protein complexes are known to be the best-studied polymeric biosrfactants (desai and Banat, 1997). Using *Acinetobacter Calcoaceticus* RAG-1, Resenberg *et al.* (1979) extracted a potent polyanioinc amphipathaic heteropolysaccharide bioemulsifier called emulsan. It is a very effective emulsifying agent for hydrocarbons in water even at a concentration as low as 0.001-0.01%. Additionally, it is note as one of the most powerful emulsion stabilzers known with the ability to resist inversion even at a water-to –oil ratio of 1:4 (Zosim *et al.* 1982) Ciriglain and Carman (1984) Synthesized liposan, and extra cellular water-solube emulsifier using Candida lipolytica. It is composed of 83% carbohydrate and 17% protein with the carbohydrate portion being a heteropolysaccharide consisting of glucose, galacotose, galactosamine and galactoronic acid. Cameron *et al.* (1988) demonstrated the production of large amounts of mannoprotein by *Saccharomyces cerevisiae.* When purified, the emulsifier contains 44% mannose and 17% protein,. The mannoprotein exhibited excellent emulsifying activity toward several oils, alkanes and

organic solvents. Other polymeric biosurfactants such as biodispersan, alas an, food emulsifiers, protein complexes and insectides emulsifiers have also been reported.

Some common structures of biosurfactants are as below.



(C)

SOPHOROLIPID

2.7 Screening of Potential Biosurfactant Producers

Methods used for screening of biosurfactants producers should be quick, reliable and assessing their potential. (Van der Vegt *et al.*, W., H.C.Vander Mei, J.Noodnams, and H.J.Busscher.1991) developed an axisymmetric drop shape analysis (ADSA) by profile for the assessment of potential biosurfactant producing bacteria. In this procedure, drops of culture broth are placed on fluoro-ethylene-propylene surface and the profile of the droplet is determined with contour monitor. Surface tensions are calculated from the droplet profile by ADSA. Only biosurfactant-producing bacterial suspensions show reduction in surface tension (Shulga *et. al.*1993). Colorimetric estimation of biosurfactants based on the ability of the anionic surfactants to react with the cationic indicator to form a coloured complex. Other simple methods include (i) a rapid drop-collapsing test (Jain D.K., D.L.C.Thompson, H.leeandJ.T. Trevors.1991), in which the drop of a cell suspension is placed on an oil-coated surface, and drops containing biosurfactants collapse where as nonsurfactant-contating drops remain stable; . (ii) A direct thin layer chromatographic technique for raid characterization of biosurfactant producing bacterial colonies as described by (Matsuyama *et al.* 1991). (iii) Colorimetric methods describe by (Sigmund and Wagner 1991 and Hansed *et al.* 1993) for

careening of rhamnolipid-producing and hydrocarbon-degrading bacteria, respectively; and (iv) estimation of the emulsification index value (E 24) by vigorously shaking culture broth samples with and equal volume of kerosene and measuring the present emulsification after 24 H by the method of cooper and Goldenberg 1987, which is most suitable for emulsifying biosrfactants.

2.8 Biosynthesis of biosrufactant and its regulation

Tow primary metabolic pathways, namely, hydrocarbon and carbohydrate, are involved in the synthesis of their hydrophobic and hydrophilic moieties, respectively. The pathways for the synthesis of these tow groups of precursors are diverse and utilize specific sets of enzymes. In many cases, the first enzymes for the synthesis of these precursors are regulatory enzymes; therefore, in spite of the diversity, there are some common features of their synthesis and regulation. The detailed biosynthetic pathways for the major hydrophobic and hydrophilic moieties have been extensively investigated and are well documented; however, a brief account by Hommel and Ratledge (Hommel, R.K., and C. Ratledge. 1993) may be useful.

The hydrophilic and hydrophobic moieties of biosurfactants are synthesized by two metabolic pathways: the hydrocarbon and carbohydrate pathways (Desai and Banat, 1997), According to Syldatk and Wagner (1987), who documented various possibilities for the synthesis of different moieties of biosurfactants and their association, any of the following possibilities could occur:

(a) Both Hydrophilic and hydrophobic moieties are synthesized de novo by two independent pathways,
(b) The hydrophilic moiety is synthesized de novo while synthesis of the hydrophobic moieties induced by the substrate,
(c) The hydrophobic moiety is synthesized de novo while synthesis of the hydrophilic moieties induced by the substrate,
(d) Both hydrophilic and hydrophobic moieties have substrate-dependent synthesis.

The regulation of biosurfactant production is generally governed by there main mechanisms, namely induction, repression and nitrogen and multivalent ions.

Induction of biosrufactant synthesis can be achieved by addition of long chain fatty acids, hydrocarbons or glycosides to the growth medium; while addition of D-glucose, acetate or tricarboxylic acids represses their production. The induction of sophorolipid synthesis by addition long chain fatty acids, hydrocarbons, of ghycerides to the growth medium of *Torulopsis magnoliae* (Tulloch, A. P., J. F. T. Spencer, and P. A. J. Gorin. 1962), of trehaolpid synthesis in *Rhodococcus erytheropolis by* addition of hydrocarbons. (Rapp, P., H. Bock, V .Wray and F.Wagner.1979. Rapp, P. H.Bock E. Urban, F. Wagner, W. Gebetsberger, and W. Schulz, 1997), and of glycolipid-EM in *p. aeruginosa* SB-30 by addition of alkanes (Chakrabary, A. M. 1985) has been reported. The inducible nature of biosurfactant in *Endomycopsia lipolytica* has also been confirmed (Roy, P.K., H. D. Singh, S. D. Bhagat, and J. N. Baruah. 1979) Induction also appears to be the general regulatory mechanism used to control the onset synthesis of most lipopeptide biosurfactants (Besson, F, and G Michel. 1992 Kluge, B., J. Vater, J. Sainikow, And K. eckart, 1989, Ullrich, C., Z. Palacz, and J. Vater. 1991). Nitrogen of metal ion dependent regulation also played a prominent role in biosurfactant synthesis. It was observed that rhamnolipid synthesis in *Pseudomonas aeruginosa* occurs upon exhaustion of nitrogen and commencement of the stationary phase of growth (Guerra-Santos *et al.* 1984.; Ramana and Karanth, 1989). Lastly, the limitation of multivalent cations caused overproduction of biosurfactants. The limitation of multivalent cations caused overproduction of bio-surfactants. The limitation of multivalent cations also causes overproduction biosurfactants (Guerra-Santos, L. H., O. Kaappeli, and A Fiechter. 1984. Itoh, S., and T. Suzuki. 1974. Syldatk, C., S. Lang, U. Matulovic, and F. Z. Wagner. 1985) Guerra-Satos *et al.* (1986) Demonstrated that by limiting the concentrations of salts of magnesium, calcium, potassium, sodium, and trace elements, a higher yield of rhamnolipid can be achieved in *P. aerations* DSM 2659 . Iron limitation stimulates

biosurfactant production in *P. flourescens* (Presson, A.,G. Molin, and C. Weibull., N. Anderson, and J. Sjoholm.1990) and *P. aeruginosa* (Guerra-Santos, L. H., O. Kappeli, and A Fiechter. 1984, 1986), where as addition of iron and manganese salts stimulates biosurfactant production in both *B. subtitles* (Cooper , D.G.,C. R. Mac Donald, S. J. B. Duff, and N. Kosaric. 1981) and *Rhodococcus sp.* (Abu-ruwaida, A. S.,I.M. Banat, S. Haditirto, and a. Khamis. 1991.)

2.9 Bio-process development: Optimum production and recovery

An efficient and economical bioprocess is that foundation fro every profit making biotechnology industry. Hence bioprocess development is the primary step towards commercialization of all biotechnological products, including biosurfactants. Any attempt to increase the yield of a biosurfactant demands optimal addition of media components and selection of the optimal culture conditions that will induce the maximum or optimum productivity. Similarly, efficient downstream processing techniques and methods are needed for maximum product recovery.

Table 2. Various biosurfactants produced by microorganisms

Microorganism	Type of surfactant
Torulopsis bombicola	Glycolipid (sophorose lipid)
Pseudomonas aeruginosa	Glycolipid (rhamnose lipid)
Bacillus licheniformis	Lipoprotein (?)
Bacillus subtilis	Lipoprotein (surfactin)
Pseudomonas sp. DMS 2847	Glycolipid (rhamnose lipid)
Arthrobacter paraffineus	Sucrose and fructose glycolipids
Arthrobacter	Glycolipid
Pseudomonas flurescens	Rhamnose lipid
Pseudmonas sp. MUB	Rhamnose lipid
Torulopsis petrophilum	Glycolipid and/or protein
Candida tropicalis	Polysaccharide-fatty acid complex
Corynebacterium lepus	Corynomycolic acids
Acinetobacter sp. HO1-N	Fatty acids, mono-and diglycerides
Acinetobacter calcoaceticus Rag-1	Lipoheteropolysaccharide (Emulsan)
Acinetobacter calcoaceticus 2CAC	Whole cells (lipopeptide)
Candida lipolytica	»liposan« (mostly carbohydrate)
Candida petrophilum	Peptidolipid
Nocardia erythropolis	Neutral lipids
Rhodococcus eryithropolis	Trehalose dimycolates
Corynebacterium salvonicum SFC	Neutral lipid
Corynebacterium hydrocarboclastus	Polysaccharide-protein complex

2.10 Factors affecting on biosurfactant production

Various factors must be taken into consideration during set up of microbial biosurfactant production. Production economy is the major bottleneck in biosurfactant production, as in the case with most biotechnological process.. Often the amount and type of a raw material can

contribute considerably to the production cost. It is estimated that raw material accounts for 10-30% of total production cost.

2.10.1 Carbon source

Water-soluble carbon sources such as glycerol, glucose, mannitol, and ethanol were all used for rhamnolipid production by *pseudomonas spp*. Biosurfactant product, however, was inferior to that obtained with water immiscible substrates such n-alkanes and olive oil (Rebert, M.,M.E. Mercade, M.P. Bosch, J.L.Parra, M.J. Espuny. M. A.)

Manresa and J. Guinea. 1989, Syldatk, C.S. Lang U. Matulovic and F. Z. Wagner. 1985, Yamaguchi, M.A. Sato, and A. Yakuyama. 1976). Syldatk, C.S. Lang and F. Wagner U. Matulovic 1985 and Edmonds, P, and J.J. Cooney. 1969, demonstrated that although different carbon sources in the medium affected the composition of biosurfactant production in *pseudomonas spp.* When *Arthobacter paraffineus*. ATTC 19558 was grown on D-glucose, supplementation with hexadecane in the medium during the stationary growth phase resulted, in a significant increase in biosurfactant yield (Duvnjak, Z., D. G. Cooper, and N. Kosaric 1982). Duvnjak and Kosaric 1985, showed the presence of large amounts of biosurfactant bound to *Corynebacterium. lepus* cells when grown on glucose, and addition of hexadecane facilitated the release of surfactant from cells. Glycolipid production by *T. bomibcola* is stimulated by the addition of vegetable oils during growth on 10 % D-glucose medium, giving a yield of 80 g. z. liter21 (Asmer, H. J., S. lang, F. wagner, and V. Wrey 1988. cooper, D. G., E.R.A. Eccles, and J. D. Sheppard, 1988). Davila *et.. al.* 1992, demonstrated a high yield of sophorse lipids by overcoming product inhibition in *Candida bombicoal* CBS 6009 through the addition of ethyl esters of repessed oil fatty acids in D-glucose medium. Using *T. apicola* IMET 43747, Stuwer, O., R., Hmmel, D. Haferburg, and H.P. Kieber. 1987, achieved glycolipid yields as high as 90 g z liter 21 with a medium containing D-glucose and sunflower oil. In an interesting study, Lee and Kim (1993) reported that in batch culture, *T. bombicola* channeled 37 % of the carbon input to produce 80 g of sophorolipid per liter.

Armin fiechter (1992) developed integrated system for biosurfactant synthesis by *pseudomonas fluorescens* up to 10 g 1-1 h-1 in batch fermentation. Hamme and ward (2001) reported physical and metabolic interactions of *pseudomonas sp.* Strain JA5-B45 and *Rhodococcus sp.* Strain F9-D79 during the growth on crude oil. Dynamic emulsion was formed by strain JA5-B45, which indirectly increased crude oil availability for strain JA5-B45, which indirectly increased crude oil availability for strain JA5-B45.

Kinetc study or fermentative biosurfactant production by lactobacillus strain was reported by L. Redrigues *et. al.* 2006. Screening of four *lactobacillus* strain was performed and shown that biosurfactant production occurred mainly in the first four hours.

Physicochemical and functional characterization of biosurfactant produced by *Lactobacillus lactis* 53 was also reported by L. Rodrigues *et. al.* 2006.

Optimization of the medium for biosurfactants production by probiotic bacteria was carried out using response surface methodology, biosurfactant produced by *Streptococcus thermophilus* A was studied by L. Rodriguez *et al.* 2006. A fraction rich in glyoclipid was obtained. Critical micelle concentration achieved was 20 g/1 allowing for surface tension value of 36 mJ/m2

Promising strain *Candida glabrata* isolated form mangrove sediments, was found capable of producing extra cellular water-soluble emulsifying agent, and identified as heteropolymer. Maximum emulsifier production was observed when culture grown on soluble and insoluble substrates cotton seed oil plus glucose, reaching values of 10.0g/1 after 144 hours at 200rpm. (L. Sarubbo *et al.* 2006) Bioemulsifier constituted of carbohydrate, protein and lipids was produced by *Candida lipolytica* using D-glucose and babassu oil as carbon sources. Emulsification activity reported was 0.666 units (M. H. V. Harrop *et al.* 2003).

Favobactrium sp produced biosurfactants when grown on used oils as substrate. Biosurfactants found biodegradable and lowered surface tension of water to 27 dynes/cm. (Shyh-yau Wang and C. Vipulanandan, 2006).

Productions of biosurfactants using substrates from renewable resources were studied (Suppasil Maneerat, 2004)Mercade *et al.* 1993 found that *pseudomonas sp*. JAMM could reduce the surface tension of culture medium comprising 100g/1 olive oil mill effluent (OOME). Deshpande and Danniels (1995) used animal fat for the production of biosurfactant by *Candida bombicola.*

Haba *et al.* (2000) reported microorganisms are able to grow on frying oil and produced products with potential industrial application such as lipase, molasses, whey and starch rich wastes were reported as substrate for biosurfactant production (Lazaridon *et al.* 2002: Lee *et al.* 2003; and Barnett *et al.* 1999)

2.10.2 Nitrogen Source

Biosurfactant production is also affected by medium constituents other than carbon sources. Among the inorganic salts tested, ammonium salts and urea were preferred nitrogen sources for biosurfactant production by *Arthrobacter parafineus* (Duvnjak, Z.,D., G. Cooper, and N. Kosaric. 1993), where as nitrate supported maximum surfactant production in *P. aeruginosa* (Guerra-Santos, L. H. O. Kappeli, and A Fiechter. 1984 MecElwee, C. G., H. Lee, and J. T. Trevors. 1990 rebert, M.,M. E. Mercade, M. P. Bosch, J. L. Parra, M. J. Espuny, M. A. Manresa, and J. Guinea. 1989) and *Rhodococcus spp.* (Abe-Ruwaida, A. S., I. M. Banat, S. Haditirto, and A. Khamis.1991). Biosurfactant production by *A. paraffineus* is increased by the addition of L-amino acids such as aspartic acid, glutanmic acid, aspargine, and glycine to the medium (Duvnjak, Z., D. G. Cooper, and N. Kosaric. 1983) Robert *et al.* (1989) and Abu-Ruwalda *et al.* (1991) observed nitrate is the best source of nitrogen for biosurfactants production by *pseudomonas strain* 44T1 and *Rhodococcus strain* ST-5 growing on olive oil and paraffin, respectively. The production started after 30 h of growth, when the culture reached nitrogen limitations, and continued to increase up to 58 h of fermentation. Guerra-Santos, L. H., O. Kappeli, and A. Fiechter. 1984, 1986 showed maximum rhamnolipid production after nitrogen limitation at a C: N ratio of 16:1 to 18:1 and no surfactant production below a C: N ratio of 11:1 where the culture was

not nitrogen limited. According to Hommel *et al.* (1987), the concentration of hydrophobic carbon source determines the conversion of carbon available to the biosurfactant. The dimorphic Basidimycete *Ustilago maydis* produces large amounts of surface active compound under conditions of nitrogen starvation was reported by S. Hewald *et al.* 2005

Medium cheap substrates: Economical and promising alternative

2.10.3 Renewable-resources

Production economy is the major bottleneck in biosurfactant production, as in the case with most biotechnological processes. Often the amount and type of a raw material can contribute considerably to the production cost; it is estimated that raw materials account for 10-30% of the total production cost in most biotechnological processes. Thus to reduce this cost it is desirable to use low-cost raw materials (Table 2) for the production of biosurfactants 10-40. One possibility explored extensively is the use of cheap and agro-based raw materials as substrates for biosurfactant production. A variety of cheap raw materials, including plant-derived oils, oil wastes, starchy substances, lactic whey and distillery wastes have been reported to support biosurfactant production.

1. Vegetable oils and wastes.
2. Lactic whey and distillery wastes.
3. Starchy substrates.
4. Olive oil mill effluent.
5. Animal fat.
5. Soaps tock
6. Molasses.
7. Frying Oil.

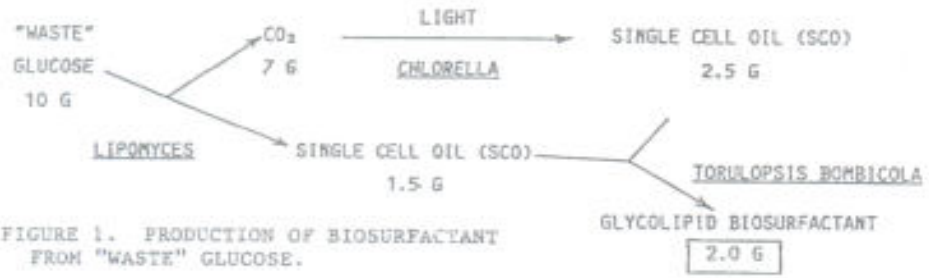


FIGURE 1. PRODUCTION OF BIOSURFACTANT FROM "WASTE" GLUCOSE.

2.11 Environmental factors:-

Growth conditions and environmental factors such as pH, temperature, agitation, and oxygen availability also affect biosurfactants production through their effects on cellular growth or activity. The pH of the medium plays an important role in sophorolipid production by *T. bombicola* (Gobbert, U.S, Lnag, and F. Wagner. 1994) Rhamnolipid production in *pseudomonas spp.* Was at its maximum at a pH range from 6 to 6.5 and decreased sharply above pH 7 (Guerra Santos, L. H., O. Kappeli, and A. Fiechter. 1984) In contrast, Powalla, M., S. Lang and V. Wary. (1989) showed that penta and disaccharide lipid production in *N. Corynbacteroides* is unaffected in the pH range of 6.5 to 8. In addition, surface tension and CMCs of a biosurfactant product remained stable over a wide rang of pH values, whereas emulsification had a narrower pH range (Abu-Ruwaida, A. S., I. M. Banat, S. Haditirto, and a Khamis. 1991, S. Salem, and Pseudomonas sp. Strain DSM-2874 (Syldatk, C.,S. Lang U. Matulovic, and F. Z. Wagner. 1985), temperature causes alteration in the composition of the biosurfactant produced. A Thermophilic *Bacillus sp.* Grew and produced biosurfactant at temperatures above $40^{.0}$ C. (Banat, I. M. 1993). An increase in agitation speed results in the reduction of biosurfactant yield due to the effect of shear in *Nocardia erythropolis* (Margaritis, A., K.Kennedy, and J. E. Zajic.1979). While studying the mechanism of biosurfactants production in *A. Calcoaceticus* RAG-1, Wang and Wnag (1990) revealed that the cell bound polymer/dry-cell ratio decreases as the shear stress increased. On the other hand in yeast biosurfactant production increases when the agitation and

aeration rates are increased (Spencer, J. F. T., D. M. Spence, and A. P. Tulloch, 1979). Sheppard and Cooper (1990) have reported that surfactin production by *Bacillus subtilis,* Oxygen transfer is one of the key parameters for the process optimization and scale-up during process development.

2.12 Potential commercial applications

Biosurfactants have become recently an important product of biotechnology for industrial and medical applications. Thereason for their popularity, as high value microbial products, is primarily in their specific action, low toxicity, relative ease of preparation and widespread applicability. They can be used as emulsifiers, wetting agents, spreading agents, functional food ingredients and detergents in various industrial sectors such as petroleum and beverages, cosmetics and pharmaceuticals, organic chemicals, agrochemicals and fertilizers, Environmental and management and many others.

The unique properties of biosurfactants allow their use and possible replacement of chemically synthesized surfactants in great no.of industrial operations. Surfactants are used by many industries and one could easily say that there is almost no modern industrial operation where properties of surface and surface active agents are not exploited. The potentials application of biosurfactants in industries is also reality. The properties of interest of an ideal biosurfactants are

1. In changing surface active phenomenon as lowering surface and interfacial tension.
2. wetting and penetrating actions
3. Spreading
4. Hydrophylicity and hydrophobicity actions.
5. Microbial growth enhancement.
6. Metal sequestration
7. Antimicrobial action.

Most of the applications today involve the use of chemically synthesized surfactants. Production of surfactants in united states and worldwide is

estimated at 3.4 X 10^9 kg and 7 x 10^9 kg in 1989 respectively. The U.S. surfactant industry shipments were $ 3.65 billion in 1989. The applications are very wide in variety of industries as shown in table below.

The advantages of biosurfacants, compared to their chemically synthesized counterparts are:

- Biodegradability
- Generally low toxicity
- Biocompatibility and digestibility- which allows their application in cosmetics, pharmaceuticals and as functional food additives.
- Availability of raw materials – biosurfactants can be produced from cheap raw material which are available in large quantities; the carbon source may come from hydrocarbons, carbohydrates and / or lipids, which may be used separately of in combination with each other
- Acceptable production economics – depending upon application, biosurfactant can also be produced from industrial wastes and byproducts and this is of particular interest for bulk production for use in petroleum-related technologies.
- Use in environmental control – biosurfactants can be efficiently used in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil.
- Specificity – biosurfactants being complex organic molecules with specific functional groups are often specific in their action, demulsification of industrial emulsions, specific cosmetic, pharmaceutical, and food applications.
- Effectiveness –at extreme temperature, pH and salinity.

A number of applications of biosurfactans have been researched into and published. Its usefulness to man in most aspects of human life can not be over emphasized. The enormous market demand for surfactants is currently met numerous synthetic, mainly petroleum-based chemical surfactants, these compounds are usually toxic to the environment and as well as been non-

biodegradable. Further more, they may bio-accumulate and their production, processes and by-products can be environmentally hazardous. It has become necessary that tightening environmental regulations and increasing awareness for the need to protect the ecosystem have effectively resulted in an increasing interest in biosurfactants as possible alternates to chemical surfactants are beginning to acquire a status as potential performance-effective molecules in various fields. Presently, biosurfactants are mainly used in studies on enhanced oil recovery and hydrocarbon bioremediation (Rahman *et al.* 2004, 2006). The worldwide production of surfactants amounted to 17 million metric tones (t) in 200 (including soaps) with expected future growth rates of 3-4% year $^{-1}$ globally and 1.5 - 2.0% in the EU (Whalley, 1995). Industrial applications of surfactants are classified according to how they are applied. These are surfactants used in detergents and cleaners (54%); as auxiliaries for textiles, leather and paper (13%); in chemical processes (10%); in cosmetics and pharmaceuticals (10%); in the food industry (3%); in agriculture (2%) and in others (8%).

An evolution of the ability of aqueous bio-surfactant solutions showed that the bio-surfactants were able to remove significant amount of crude oil from the contaminated soil at different solution concentrations for instance rhamnolipid and SDS removed up to 80% oil and lecithin about 42 %.

Now a days increased interest in applications are involved in medical field for combating many diseases because of their

1) Antimicrobial activity of biosurfactants

The antimicrobial activity of several biosurfactants has been reported in the literature for many different applications.(Cameotra S,MakkarR.,2004) For instance, the antimicrobial activity of two biosurfactatnts obtained from probiotic bacteria, *Lactococcus lactis* 53 and *Strepococcus thermophilus* A, against a variety of bacterial and yeast strains isolated from explanted voice prostheses was evaluated, (RodrigusLR van der Mei HCTeixeria J *et al.2004)* both biosurfactants have a high antimicrobial activity even at low

concentrations against *Candida tropicalis* GB 9/9, one of the strains held responsible for prostheses failure. At the highest concentration tested both biosurfactants were active against all the bacterial and yeast strains studied. Another study suggested that biosurfactants are reliable alternative for antibiotic treatment in future. (Reid *et al 1998, 2001)*

2) Anti-adhesive activity of biosurfactants

Biosurfactants have been found to inhibit the the adhesion of pathogenic organisms to solid surfaces or to infection sites; thus, prior adhesion of biosurfactants to solid surfaces might constitute a new and effective means of combating colonization by pathogenic microorganisms (Singh P ,Cameotra S. 2004) Pre-coating viny1 urethral catheters by running the surfactin solution through them before inoculation with media resulted in a decrease in the amount of biofilm formed by *Slmonella typhimurium*, *Salmonella enterica, E. coli* and *Proteus mirabils*. (MirelesJR, Toguchi A, Harshey RM.2001) Given the importance of opportunistic infections with *Salmonella species*, including urinary tract infections of AIDS patients, these results have great potential for practical applications.

3) Biomedical and therapeutic applications of Biosurfatants

Some Biosurfatants are suitable alternative to synthetic medicines and antimicrobial agents and may be used as safe and effective therapeutic agents. (Mireles J. R., Toguchi A., Harshey R. M. 2001, Banet I.M., Makkar R. Cameotra S. 2000). There has been increasing interest in the effect of Biosurfatants on human and animal (Benincasa M, Abaloscells A., Oliveria I *et al.* 2004) and cell lines (Kitamoto D., Yanagishita H., Shinbo H. *et al.*) rhamnolipids produced by *p. aeruginosa* (Lang S Wullbrandt D. 1999, Maier R, Soberan-Chavez G. 2000) and lipopeptides produced by *B. Subtilis* (Vollenbroich D. PauliG Ozel M. *et al* 1997) and *B. lichenifromis* (Fiechter A.1992, Jenni K, Kappli O, Fietcher A 1991, Yakimov M, Timmis K, Wray V. *et al.* 1995) have been shown to have antimicrobial activates. (Jenni K., Kappli O, Fietcher A. 1991). Determined the structure and characterized surface activities of biosrfactants produced by *B. lichenifromis*, while (Lin S, Carswell

K, Sharma M 199) described their continuous production (Yakimov M, Timmis K, Wray V.*et al.* 1995), demonstrated the antibacterial activity of *lichenysin* A, a biosurfactant produced by *B. lichenifromis* that compares (favourably with other surfactants.Kosaric N1996) Possible applications of biosurfactants as emulsifying agents for drug transport to infection site, as agent supplementing the pulmonary surfactant and as adjuvant for vaccines were suggested by (Kosaric 1996).

4) As a soil bioremediation

The accumulation and persistence of toxic materials in water and soil represents a major problem because various organic chemicals generated as a byproduct from various industries should be treated properly prior to their release in water or soil, in such cases biosurfactants have been shown to promote/enhance biodegradation of hydrocarbons. Thus depredation time and particularly adaptation time, for microbe was shortened. (Naim Kosaric2002)

5).In the petroleum industries

Biosurfactants can potentially replace chemical analogue compounds, even offering additional advantages, all through the chain of petroleum processing for eg. Oil extraction, oil transportation, and oil tank or container cleaning. (Van Dyke M. I., and Trevors J.T. 1991)(Ibrahim M.Banat, Amedea Perfumo and Roger Marchant)

6) Microbial enhanced oil recovery

(Van Dyke M. I., and Trevors J.T. 1991)Reservoir rock is like a sponge that entraps and expels oils. Classical production technologies called primary and secondary recovery, can only partially recover the oil present in the field, with an efficiency estimated at one third to half the overall amount of oil available. Moreover, such efficiency is expected to decrease during the gradual depletion of light crude reservoirs leaving the viscous crude oil. This requires the development of new exploitation strategies based on the tertiary recovery processes, whose aim is to enhance oil recovery (EOR)(Planckert M2005) .Among them ,microbial enhanced oil recovery(MEOR)exploiting

microbial activities and metabolites, is presently gaining attention due to many advantages.

7) Crude oil transportation in pipeline

Oil viscosity is the major factor mainly slowed the flow in pipeline, to over come this use of bioemulsifier type biosurfactants found to be suitable application. (Ibrahim M.Banat, Amedea Perfumo and Roger Marchant)

8) Clean up of oil storage tank

In 1991 banat described application of microbial biosurfactants for clean up of oil storage tank(Banat I M. 1991).sludge and oil deposits accumulates at the bottom and walls of storage tank ,traditional methods used to clean this are hazardous ,time consuming and expensive use of biosurfactants are effective alternates in such cleaning activity . (Ibrahim M.Banat, Amedea Perfumo and Roger Marchant)

9) Use in cosmetics and food industries

Restricted data is available for use of biosurfactants in cosmetics and food industries. Some of the most commonly used cosmetic products using biosurfactants are insect repellents, bath products, antidandruff products, nail care, deodorants, contact lens solutions, tooth pastes, foot care, conditionars, moisturizers, shampoos etc. (N.Kosaric1992).

10) For crude oil contaminated soil washing

An evaluation of the ability of aqueous biosurfactant solutions for possible applications in washing crude oil contaminated soil was carried out. (Kingsley Urum, TurgayPekdemir. 2003)

CHAPTER-III

MATERIAL'S AND METHODES

3.1. MATERIALS

3.1.1 CHEMICALS

CHEMICALS used in the study of project, "studies on production of biosurfactants by alkaliphilic bacteria" are listed as in ANEXER-I

- List of CHEMICALS.

All chemical used in the study are of himedia laboratories ltd. Mumbai.

3.1.2 GLASSWARE.

- Glassware used in the present study is as in the ANNEXURE -II All glassware used are of borosil glass/borosilicate glass.

3.1.3. INSTRUMENTS.

- Instruments used ANNEXURE-III

The Instruments used in the present study listed as below

Sr. No.	Name of Instruments	Manufactures
1	pH Meter	Biocraft ,New Delhi
2	Centrifuge machine	Navin Scientific industries
3	Digital balance	K.Roy waranashi biocraft
4	Rotary shaker	New Delhi
5	Oven	
6	Vertex mixer	

3. 2 Isolation of alkaliphilic bacteria:

Sources used for isolation of Microorganisms in the present study was

1) Soil Sample

2) Water Sample

These Samples are collected from the Natural alkaliphilic environment like Lonar Lake, Lonar Dist: Buldhana (Maharashtra State)

3. 3 Collection of Samples

From above Natural Source Total **19** cultures are isolated and labeled as below

01) C_1C – 25/6
02) C_1C – 26/6
03) C_2 C - 26/6
04) C_1Ps – 23/6
05) C_1Ps – 24/6
06) C_2K
07) C_3 K
08) CRYE – C_2
09) CGM/AM/24/6
10) $CRYEMAC_1$
11) CRYEMA C_3
12) RC_3K
13) NC_2K
14) $N_2FGA\text{-}C_1$
15) C_2GG
16) C_3PS
17) C_2
18) ACT-C_1
19) PDA-F_1

3. 4 Preservation of isolates

All above isolates were preserved on the respective agar slant of pH – 10.7. The medium used for the preservation are

1) Cetrimide agar (Himedia)

2) Kings B agar (Himedia)

3) Pseudomonas agar (Himedia)

All isolates isolates were preserved in refrigerator (4^0c) after growth at room temperature (28^0c) for 24 hrs on respective agar slant.

3. 5 screening for lipolytic alkaliphilic bacteria

For screening of lipolytic bacteria above isolated 20 cultures are tested for lipolytic activity on butter fat agar for which stock cultures preserved on respective agar slant were inoculated in to nutrient broth medium (Himedia) of pH 10.7 and incubated at room temperature for 24 hrs .After growth in nutrient broth a loopful with a calibrated loop of himedia (0.005 ml) was spot inoculated in the butter fat agar (A.M. Deshmukh).

During preparation of butter fast agar 100 ml Nutrient agar of PH – 10.7 was prepared in to 250 ml conical flask and sterilized at 121^0c for 15 minutes and butter fat was sterilized separately in a measured amount (10 ml) in to a small 50 ml flask at 5 lb for 10 minutes.

After sterilization nutrient agar and butter fat was mixed properly in an aseptic conditions and poured in to a sterile Petri plates

These plates are spot inoculation for screening of lipolytic bacteria incubated at room temperature for 5 days to obtain growth and finally plates were flooded with saturated $CuSO_4$ solution for 15-20 min. Excess $CuSO_4$ was poured off and plates were observed for greenish blue zone as a indication of lipolysis. Of total 20 isolates following (six) cultures showing lipolytic activity in the form of greenish blue zone around colony are selected

Table 3.5.1

Sr.No	Culture	Zone of lipolysis in mm.	Growth at room temperature (28^0c)
01	C_2k	50 mm.	+++++
02	C3k	20 mm.	----
03	C_1C	15 mm.	++
04	C_1C./25/6	10 mm.	----
05	C_2C	17 mm.	+++
06	C_1Ps	15 mm	+

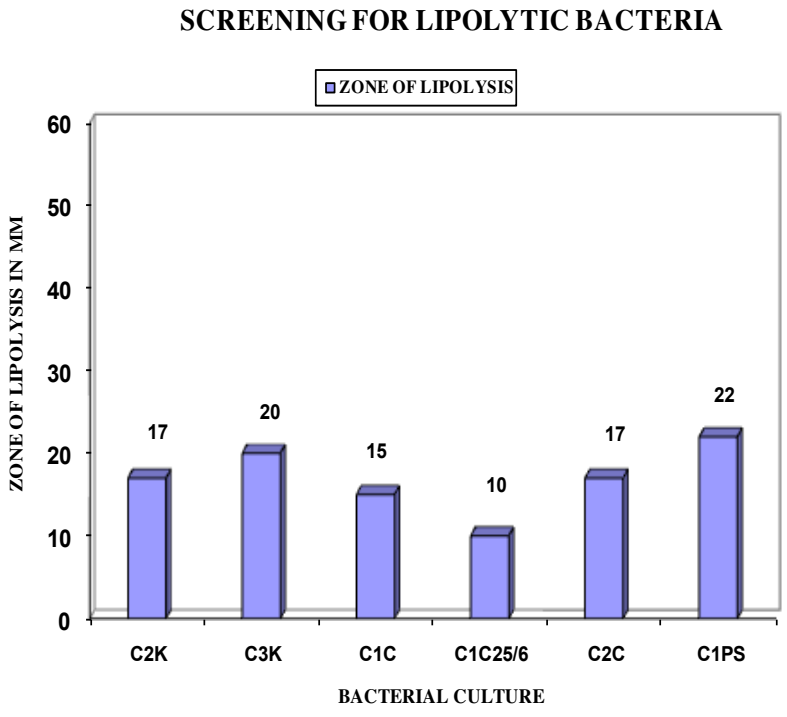


Fig: 3.5.1 screening for lipolytic bacteria.

The lipolytic activity of 6 (six) Cultures was further confirmed by observing Zone of lipolysis on Tributyrine agar medium (Himedia)

For this Tributyrine agar medium of pH – 10.7 was prepared and spot inoculated with calibrated loop (Himedia) (0.005.ml) the results on Tributyrine agar medium are as

Table 3.5.2

Sr.No.	Culture	Diameter of Zone of Lipolysis	
		After 24 hrs	After 36 hrs
01	C_1ps	16 mm.	26mm
02	C_3K	16 mm.	20 mm.
03	C_2K	12mm.	17 mm.
04	C_1C	14 mm.	25 mm.
05	C_2C	11mm	15mm

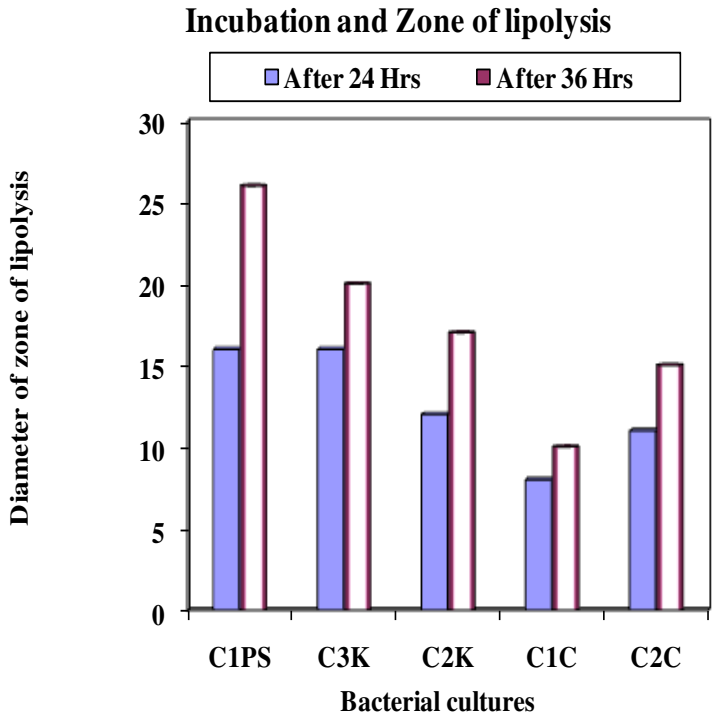


Fig.3.5.2.1screning for lipolytic bacteria on Tributyrine agar.

During screening for lipolytic bacteria effect of Tween -80 on production lipase by selected five cultures was tested and compared with zone of lipolysis on Tributyrine agar.

Enhancement of lipolytic activity was also observed and confirmed by addition of 0.5%ween - 80 in to Tributyrine agar medium of pH-10.7 and results Showing enhanced lipolytic activity by same bacterial isolates are recorded as below.

Table 3.5.3

Sr.no	**Culture**	**Diameter of Zone of Lipolysis in mm. On Tributyrine agar with 0.5 % Tween 80**	
		After24 Hrs	**After 36 Hrs**
01	C_1PS	19 mm	22 mm
02	C_3K	43 mm	50 mm
03	C_2K	22 mm	37 mm
04	C_1C	23 mm	23 mm
05	C_2C	26 mm	41 mm

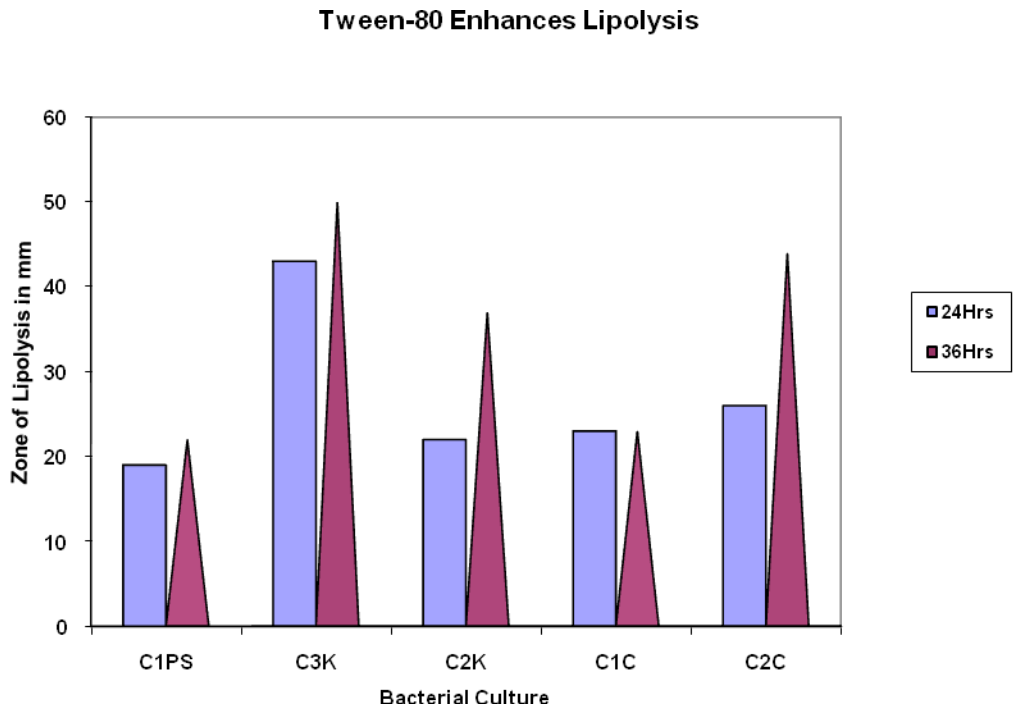


Fig: 3.5.3.1 Effect of Tween -80 on Lipase production.

3. 6 Phenotypic Identification of Isolated Bacterial Cultures

3.6.1 Grams Staining

Cultures Grown on solid media i.e. respective media (Cetrimide agar, king 'B' agar, and Pseudomonas agar) for 24 hrs to obtain active culture. Smear was prepared from the cultures (C_1PS, C_3K, C_2K, C_1C, C_2C)on a clean grease free slide and air dried the underside of the slide was passed three times over the flame or Bunsen burner to induce adherence.

Following treatment was performed

- Grams crystal violet Staining reagent – 1 min
- Wash under tap water for few seconds gently and indirectly
- Stain with Grams loidne as a mordant – 2 min
- Wash with 95% alcohol for – 6 seconds

- Wash under tap water
- Stain with saffranin 30 seconds
- Wash under the tap water

Finally the slide was dried and observed under light microscope Purple coloured cells are Gram positive and pink cells are Gram negative (Murray *et.al* – 1994). Cellular morphology was also determined.

3.6.2 Catalase Test

3% H_2O_2 Solution was taken in to the clean test tube to which growth from the sold agar medium of respective culture was added with the help of sterile nichrome wire loop. Formation of air bubbles is indication of presence of catalase enzyme (Smibrt and Krieg, 1994)

3.6.3 Biochemical Tests

Table3.6.3.1

Sr. No.	Sugar fermentation	Cultures				
		C_1PS	C_2K	C_3K	C,C	C_2C
01	1. Lactose	A	A	A	A	A
	2. Glucose	A	A	A	A	A
	3. Dextrose	A	A	No	A	A
02	Citrate utilization (kosers citrate medium)	No	No	positive after **72** hrs	No	No
03	Nitrate reduction	Positive	Positive	Positive	Positive	Positive
04	Gelatin Hydrolysis	Positive	Negative	Positive	Negative	Negative
05	Starch Hydrolysis	Negative	Negative	Negative	Negative	Negative
06	Catalase	Positive	Positive	Positive	Positive	Positive
07	Lipolytic Activity	Positive	Positive	Positive	positive	positive

3.6.3.1 Sugar fermentation

- For sugar fermentation **Neutral Red pH indicator dye** is used in peptone water.
- For citrate utilization **kosers citrate medium** (himedia) is used
- For nitrate reduction – **nitrate medium** is used
- Diphenyl amine reagent is used to detect NO_2 or NO_3 hence it is necessary to make sure presence of NO_2 in the medium by addition of Ttommsdorfs reagent.
- For gelatin hydrolysis / photolytic activity **gelatin agar** and $HgCl_2$ reagent (Frazier's reagent is used to test the gelatinase activity or Zone of clearance around colony.
- For saccharolytic activity **– starch agar** and lugol's iodine reagent is used
- For lipolytic activity **Tributyrine agar** medium is used

3.6.4 Morphological cultural characteristics of isolated cultures after 24 hrs incubation on Nutrient agar plates of pH-10.7

Table 3.6.4.1

Sr. No.	Cultural Characters	Culture				
		C_1Ps	C_3K	C_2K	C_1C	C_2C
01	Size	2 mm	2mm	Punctiform	1 mm	1 mm
02	Shape	Circular	Circular	Circular	Circular	Circular
03	Colour	white (turns yellow after 48 hrs)	white	white	white	White
04	Elevation	Raised	Raised	Raised	Raised	Raised
05	Margin	Irregular	Entire	Entire	Entire	Entire
06	Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
07	Consis tancy	Sticky	Easy	Easy	Hard	Easy
08	Gram nature	Gram Negative Rods	Gram Negative Rods	Gram Negative Rods	Gram Negative Rods	Gram Negative Rods

3.7 Screening of lipolytic Isolates for biosurfactant production

After isolation of alkaliphilic bacteria from natural alkaline environment and their screening for maximum lipolytic action the selected bacterial cultures are used to test their ability for biosurfactant production. for efficient biosufactant productions GN medium is used the composition of GN Medium (Glucose – 10 gm ,KNO_3 – 2.5 gm,KH_2PO_4-01gm,$MgSO_4$-0.5g,m, Distilled water -1000 ml , pH – 10.7)

The above GN medium was taken as 50 ml amount in to the 250 ml conical flask pH was adjusted to 10.7 with NaOH solution and sterilized at 121^0C for 15 min.

5- Sets of 50 ml medium containing 250ml flask are prepared and inoculated with 4.%(W/v) from nutrient broth previously inoculated with stock cultures from slant of the selected bacterial isolates and incubated at room temperature.

(Loopful if is inoculated in to 50 ml GN medium Growth could not occurs even after 5-6 day's incubation)

3.7.1 Incubation

All five sets of flask containing 50 ml GN medium of pH-10.7 now incubated on **Rotary shaker** adjusted to 120 rpm at Room temperature (28^0C)

Cultures are harvested after 5 days to test emulsification activity

3.7.2 Growth Assessment

Growth assessment is carried out at the end of incubation in terms of dry weight. For growth assessment and estimation of emulsification index following processes is carried out

3.7.3 Centrifugation, filtration and drying

To separate bacterial biomass from fermented broth 5 ml of 5 days grown broth culture in GN medium of pH-10.7 was centrifuged at 8000 rpm for 20 min. the supernatant was colleted separately and cell pellets was washed with sterile distilled water and finally pellets were suspended on Whattsman filler paper No 40, which was previously dried at 60^0C for 2 hours and cooled and weighed, liquid was drained off and Whattsman filler paper was again dried at 600C for 2 Hrs ,removed ,cooled and weighed. The difference between two weights gives the biomass production or growth.

Table 3.7.1

Screening of maximum lipolytic bacteria for biosurfactant production after7 day's incubation on Rotary shaker adjusted to 120 rpm.

Sr.No	Culture Type	DryWeight in Mg/100 ml	Emulsion Layer	Kerosene Layer	Emulsion Index
1	C_1Ps	250mg/100ml	19	1	19
2	C_1C	300mg/100ml	9	06	1.5
3	C_2C	50mg/100ml	3	14	0.214
4	C_2K	90mg/100ml	4	13	0.3
5	C_3K	100mg/100ml	18	01	18

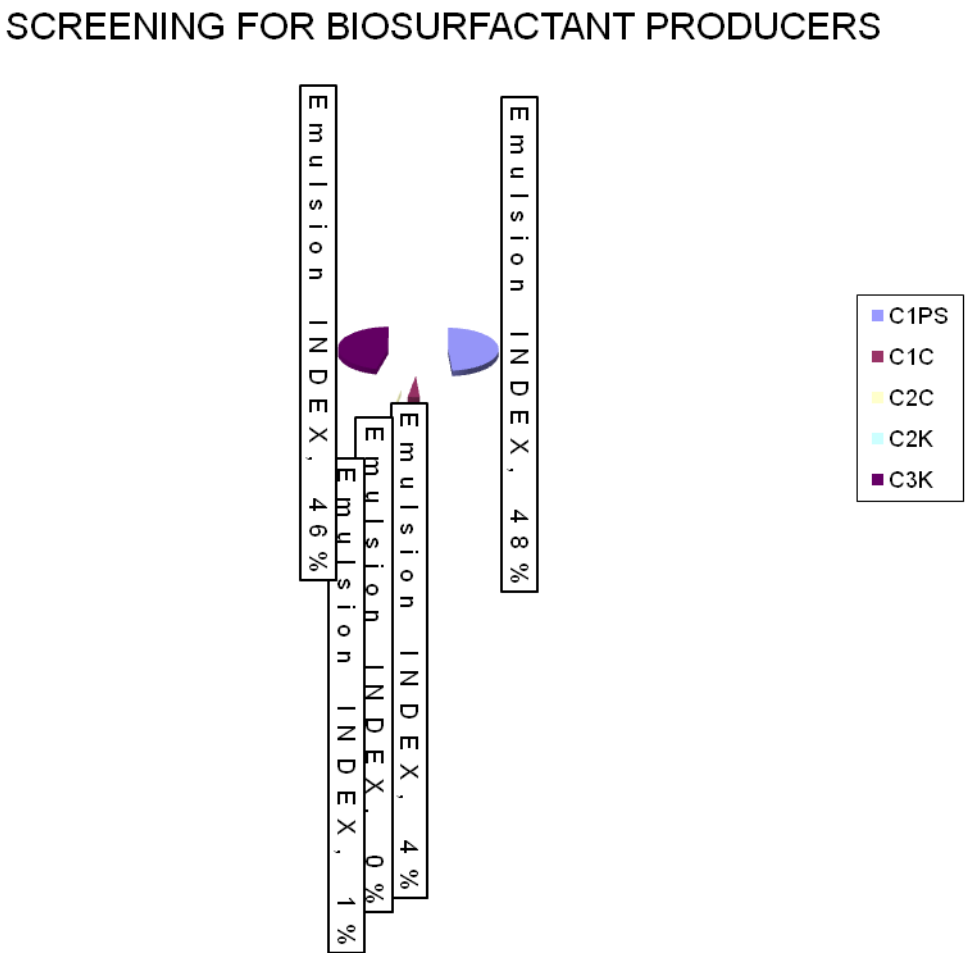


Fig: 3.7.1.1 % of biosurfactant production by lipase producing bacteria.

One the bases of biomass production and optimum emulsification activity following three bacterial isolates are selected for father study

1 C1PS

2 C_1C

3 C_3K

3.8 Growth optimization for biosurfactant production by selected bacteria

01) C1 PS

02) C_2 C

03) C_3K

Biosurfactant production is a secondary metabolism its production takes place in two different phases and is directly related to the growth of candidate organism/ bacterium because of this fact it is important to find out suitable combination of environmental conditions like pH, Temperature, substrate concentration, incubation period and aeration for optimum growth and biosurfactant production.

To standardize all conditions which affects on growth and biosurfactant production experiments are carried out in a 250 ml conical bottom flask. For this experimentation or growth condition optimization 50 ml GN medium is used as in the previous section during selection of maximum biosurfactant producing alkaliphilic bacteria following producers is used.

1 Inoculation of 50 ml GN medium with respective bacterial culture i.e. C1PS,C1Cand C3K (2% v/v inoculums is used i.e. 24 hrs growth culture is used as inoculums)

2 Filtration by using centrifugation at 5000 rpm for 20 minutes .

3 After filtration cell biomass was obtained by drying cells i.e. pellets was washed with distilled water and suspended on Whattsman filter paper No. 40.

4 Measurement of emulsification activity by kerosene emulsification method (singer N.E 1985)

3.8.1 Effect of pH:-

The effect pH on growth and biosurfactant production or emulsification activity of bacterial cultures C1PS (*Paenibacillus.sp.*) C1Cand C3K was determined by growing these bacterial cultures in GN medium for 5 day's in static manner at pH range of 10.7,to5., i.e. 10.7 7:00 and 5.0.

The results are shown in table **3.8.1.1** from this table it was found that pH 10.7 is the only pH found to be most effective for growth and surfactant production or emulsification action of the selected candidate bacterium

Table No 3.8.1.1

Effect of pH on growth and biosurfactant production or emulsification activity

pH	**Bacterial isolates**								
	C1PS			**C_1C**			**C_3K**		
	5	**7**	**10.7**	**5**	**7**	**10.7**	**5**	**7**	**10.7**
Dry. Weight mg/100ml	No growth	150mg/100 ml	200mg 100ml	No growth	No growth	300 mg/ 100ml	No growth	No growth	100 mg/ 100 ml
Emulsion Index	0.0	0.00.	20.	0.06	0.06	2.6	2.6	0.05	20

3.8.2 Effect of temperature:-

The selected bacterial isolates were incubated at temperatures 28^0C (Room temp) 37^0C and 40^0C for 5 day's in static manner in incubator having adjustment facility for desired temperature results of the experimentation were as shown in table No.3.8.2.1.

From this it was found that the optimum temperature for biosurfactant production was found to be 28^0C or Room temp.

Table 3.8.2.1

Effect for Temperature on growth and biosurfactant production by bacteria C1PS, C_1Cand C_3K

	Isolates								
Factor	C1Ps			C_1C			C_3K		
Temp-erature	28^0C R.T Shaker	37^0C	40^0C	28^0C R.T. Shaker	37^0C	40^0C	28^0C R.T. Shaker	37^0C	40^0C
Dry weight In mg/100 ml	200mg/ 100ml	150mg/ 100ml	No growth	300mg /ml	200mg /100ml	No growth	150mg /100ml	100mg/100 ml	No growth
Emulsion Index	20	18	00	2.6	2.0	00	20	16	00.

3.8.3 Effect of Incubation Period:-

For the study of Effect of Incubation period on selected bacterial isolates selected bacterial isolates were grown in GN medium with 1% Glucose at pH – 10.7 and incubated at 28^0C on Rotary shaker adjusted to 120 rpm while one set of same culture was incubated at Room temp in static manner

All these cultures after inoculation in GN medium Incubated at respective sites for 4 to 12 day's and results in the form of emulsification activity ,after different incubation period was noted

The results of this experimental set up are shown in table 3.8.3.1

Table 3.8.3.1 Effect of Incubation period

Incubation Period (Days)	Bacterial Isolates											
	C_1Ps				C_1C				C_3K			
	shaker		Static		Shaker		static		shaker		static	
	Day Wt.	Emulsion Index.	Dry Wt.	E.I	Dry Wt	E.I	Dry Wt	E.I.	Dry Wt	E.I	Dry Wt	E.I.
5. day's	150 Mg/ 100ml	18	00	00	265 Mg/ 100ml	2.1	No growth	00	130 Mg/ 100ml	18	No growth	00
06 day's	165 Mg/ 100ml	18	00	00	280 Mg/ 100ml	2.3	No growth	00	145 Mg/ 100ml	18	No growth	00
07 day's	200 Mg/ 100ml	20	00	00	300 Mg/ 100ml	2.5	No growth	00	150 Mg/ 100ml	19	No growth	00
08 day's	200 Mg/ 100ml	20.	00	00	300 Mg/ 100ml	2.5	No growth	0.0	150 Mg/ 100ml	20.	No growth	00
09 day's	200 Mg/ 100ml	20	00	00	300 Mg/ 100ml	2,5	No growth	00	160 Mg/ 100ml	20	No growth	00
10 day's	205 Mg/ 100ml	19	00	00	310 Mg/ 100ml	2.4	No growth	00	160 Mg/ 100ml	19	No growth	00
11 day's	200 Mg/ 100ml	19	00	00	310 Mg/ 100ml	2.4	No growth	00	150 Mg/ 100ml	18		00
12 day's	200 Mg/ 100ml	18	00	00	300 Mg/ 100ml	2.4	No growth	00	150 Mg/ 100ml	18	No growth	00

3.8.4 Effect of substrate concentration

To test the effect of substrate concentration on growth and emulsification activity of selected bacterial isolates (C1Ps, C1C and C_3K) these cultures were grown in G.N medium (pH – 10.7) with different concentration of glucose ranging form 0.5 gm/100ml,1gm/100ml,1.5gm/100ml and 2gm/100ml all selected bacterial cultures were inoculated in to 50ml GN medium of respective glucose Concentration and incubated on rotary shaker for 07 day's at 28^0C or Room temperature.

The results of the experimental set up are recorded in table 3.8.4.1

From results it is concluded that GN medium contain 1.5 % sugar (glucose) concentrations is optimum for growth and emulsification activity or biosurfactant production.

Table 3.8.4.1 Effect of substrate concentration:

Growth and emulsification action or biosurfactant production by selected bacterial isolates incubated on rotary shaker adjusted to 120 rpm at 28^0C

Substrate Concentration Gms/100ml Or (w/v)	C_1Ps		C_1C		C_3K	
	Dry Weight In Mgl/100 ml	Emulsion Index	Dry Wt. in Mg/100 ml	Emulsion Index	Dry Wt. in Mg/100 ml	Emulsion Index
0.5%	160	13	205	1.4	100	15
1.0%	200	20	300	2.6	150	20
1.5%	210	20	300	2.5	160	20
2.0%	220	19	305	2.5	160	18

3.8.5 Effect of nitrogen sources:-

Effect of nitrogen source on growth and emulsification activity or biosurfactant production was studied by allowing selected bacterial isolates (C1Ps ,C1 C and C_3K) to grow in G.N medium with 1% glucose with different nitrogen sources potassium nitrate ,ammonium nitrate ,Yeast extract, and urea.

After preparation of GN medium with varying nitrogen source selected cultures were inoculated with 2 v/v in a 50 ml GN medium of pH 10.7 in a 150 ml conical flasks and flasks were incubated on rotary shaker adjusted to 120 rpm at room temperature for 8day's and result in the form of dry weight per 100 mg and emulsion activity was recorded in table No 3.8.5.1.

Table 3.8.5.1 Effect of Nitrogen Sources on Growth and emulsification activity

Nitrogen Source	Bacterial isolates					
	C_1PS		C_1C		C_3K	
	Dry. Wt. In Mg/100ml	Emulsion Index	Dry Wt. In Mg/100ml	Emulsion Index	Dry. Wt. In Mg/100ml	Emulsion Index
Potassium Nitrate (KNO_3)	210mg/100ml	19	290mg/100ml	2.5	125mg/100ml	12
Ammonium Nitrate	190mg/100ml	13	245mg/100ml	2.0	105mg/100ml	09
Yeast extract	140mg/100ml	11	230mg/100ml	1.0	85mg/100ml	07
Urea	129mg/100ml	09	195mg/100ml	1.9	87mg/100ml	09

3.8.6 Effect of concentration of Nitrogen Source

To test the effect of Nitrogen concentration the selected bacterial isolates were allowed to grow in GN medium with 1% glucose of pH 10.7 Containing potassium nitrate ranging form 0.1% to 03% the isolates were allowed to grow in 50ml GN medium in 150 ml flask incubated on rotary shaker adjusted to 120 rpm at 28^0C for 8day's

Results of the experimental set up are recorded in table 3.8.6.1

Table No. 3.8.6.1

Effect of Nitrogen concentration on Growth and biosurfactant production measured in terms of emulsification activity.

Concentration Of nitrogen source (%w/v)	C1PS		C1C		C3K	
	Day weight Mg/100ml	Emul-sion Index	Dry weight Mg/100ml	Emul-sion Index	Day weight Mg/100ml	Emul-sion Index
0.1	190mg/100ml	15	270mg/100ml	1.5	90mg/100ml	08
0.5	170mg/100ml	17	270mg/100ml	1.5	110mg/100ml	08
2.0	170mg/100ml	17	295mg/100ml	2.0	125mg/100ml	11
2.5	205mg/100ml	19	300mg/100ml	2.5	140mg/100m	13
3.0	210mg/100ml	18	300mg/100ml	2.5	140mg/100ml	13

3.8.7 Effect of Aeration:-

To test the effect of aeration on growth and biosurfactant production the selected bacterial isolates were grown in GN medium with 1 % glucose of pH-10.7 the cultures were incubated on rotary shaker adjusted to 120 rpm at 28^0C for 8 day's

Result of experimental set up were recorded in the table 3.10 one set of same(28^0C) room temperature and results are recorded in table 3.8.7.1.

Table No 3.8.7.1

	Bacterial isolates					
	C1PS		C1C		C_3K	
	Dry weight Mg/100ml	Emulsion Index	Dry weight Mg/100ml	Emulsion Index	Dry weight Mg/100ml	Emulsion Index
Culture on Rotary shaker	205mg/ 100ml	19	280mg/ 100ml	2.0	110mg/ 100ml	11
Static Culture	80mg/ 100ml	09	190mg/ 100ml	00	No growth	00

From all these experimental set up for growth and biosurfactant production optimization following values are concluded as optimum conditions and are used for further study

01) pH -10.7.

02) Temperature of incubation -28^{0}C.

03) Incubation period -07 Days.

04) Substrate concentration (GN /100ml) -1.5gm/1000ml.

05) Nitrogen source concentration -2.5 gm/100ml.

06) Aeration/static culture-on Rotary shaker/suspension culture.

All above values are considered to be optimum for further study.

3.9 Identification of culture

From cultures used for above study i.e.C1PS, C_1Cand C_3K **C1PS** shows maximum biosurfactant production was grown on pseudomonas agar (Himedia) and is identified by 16S rRNA sequencing and found to be *Paenibacillus sp.*

The 16SrRNA gene sequence for C1PS culture is as

>C1PS_536_F

GGGCGTAAGCGCGCGCAGGCGGTTCTTTAAGTCTGGTGTTTAAACC
CGAGGCTCAACTTCGGGTCGCACTGGACACTGGGGAACTTGAGTGC
AGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGATATGCGTAGAT
ATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGGCTGTAACT
GACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTAGGGGTTT
CGATACCCTTGGTGCCGAAGTTAACACATTAAGCATTCCGCCTGGG
GAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGACCC
GCACAAGCAGTGGAGTATGTGGTTTAATTCG**AAGCAACG**CGAAGA
ACCTTACCAAGTCTTGACATCCCTCTGAATCCTCTAGAGATAGAGGC
GGCCTTCGGGACAGAGGTGACAGGTGGTGCATGGTTGTCGTCAGCT
CGTGTCGTGTGGATGTTGGGGTGAGTCCCGCAACGAGCGCAACCCC
TGATTTTAGTTGCCAGCACTTCGGGTGGGCACTCTAGAATGACTGCC
GGTGACAAACCGGAGGAAGGCGGGGATGACGTCAAATCATCATGCT
CCTTATGACTTGGGCTACACACGTACTACAATGGCTGGTACAACGG
GAAGCGAAGCCGCGAGGTGGAGCCAATCCTATAAAAGCCAGTCTCA
GTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATTGCT
AGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTT
GTACACACCGCCCGTCACACCACGAGAGTTTACAACACCCGAAGTC
GGTGGGGTAACCCGCAAGGGAGCCA

ANNEXURE –IV

MEDIA

1. Medium used for preservation of stock cultures.

    4) Cetrimide agar (Himedia)
    5) Kings B agar (Himedia)
    6) Pseudomonas agar (Himedia)

2. Butter fat agar used for screening of lipolytic microorganisms.

COMPOSITION OF BUTTER FAT AGAR:

Sterile nutrient agar – 100 ml

Sterile butter fat – 10 ml

pH – 10.7.

3. Tributyrine agar (himedia)

Composition:

CHAPTER IV

STUDIES ON PRODUCTION AND CHARACTERIZATION OF BIOSURFACTANT BY *Paenibacillus* species (C1PS).

4.1 INTRODUCTION

Scale up of biosurfactants for industrial production is still challenging since the composition of final products is affected by the nutrient. Micronutrient and environmental factors, it is important to find out the right surfactant for industrial scale up (Pattanathu K.S.M, Rahman and Edward Gakpe U.K-2008).

Successful commercialization of every biotechnological product depends largely on its bioprocess economics. At present the prices of microbial biosurfactant are not competitive with those of the chemical surfactants due to their high production cost and low yields. This is the main reason that they have not been commercialized extensively for the production of commercially valuable biosurfactants, process optimization at the biological and engineering level needs to be improved. Improvement in production technology already enabled 10-20 fold increase in productivity (Krishnaswamy Muthusamy, Subbucheffiar Gopalakrishna, Thiengungul Kochupappy Ravi and Panchaksharam Sivachidambaram-2008)

To overcome non-economical large scale production of biosurfactant inexpensive substrate and effective microorganism has to be intensively developed.

At present only minor fraction of extremophilic microorganisms have been exploited for cultivation and production. More ever very less attention has been directed in the development of processes for biosurfactant production by extremophiles.

In this study alkaliphilic bacteria paenibucillus spp (C1PS) has been (Used to find) tried as extremophile to find out their ability for the production of biosurfactant using the GN medium.

Now a day there is an increased interest in use of extremophiles for biotechnological production, hence this is an effort to fulfill this demand.

Use of alkaliphilic bacteria has very little tried before for production of biosurfactant.

4.2 MICROORGANIM

Alkaliphilic bacteria *paenibacillus. sp.* (C1PS) was isolated from the natural alkaline environment by standard microbiological methods as on P.No….. And after their identification by 16S rRNA sequencing is carried out and these bacteria are used in the present study of biosurfactant production.

4.3 PRODUCTION MEDIUM / CONDITIONS

The modified G.N. Medium with composition, glucose-2gm, KNO_3-2.5gm, KH_2PO_4-1gm, $MgS0_4$-05gm in 1000 ml. distilled water, pH of the medium was adjusted to 10.7 and Sterilized at 121^0 c for 15 min.

This sterile modified GN medium was used for the study.

In 250 ml Erlenmeyer fiask 100 ml modified GN medium of pH 10.7 was inoculated with 5% inoculum of *paenibacillus sp.* (C1PS) grown in nutrient broth of PH 10.7 for 24 hrs. These inoculated flasks now incubated on rotary flask shaker adjusted to 120 rpm at room temp (28^0c-30^0c) for 7 days.

Results in the Table - 4.1 Shows that modified GN medium was suitable for growth and biosurfactant production by *paenibacillus SP* (C1PS).Growth and biosurfactant production of *paenibacillus SP* (C1PS) in GN medium.

Table No. 4.1

Culture	Dry Weight	Emulsion Index
Paenibacillus sp(C1PS)	200 mg/100 ml	20.0

.

After 7 days when maximum growth and biosurfactant production confirmed, flasks has been removed from the rotary flask shaker and biosurfactant was extracted using solvent extraction method.

For recovery of biosurfactant, solvent extraction was done in the solvent system chloroform, diethyl ether and Hexane. Solvent extraction of

biosurfactant was carried out by centrifugation of culture medium (Fermented GN medium for 7 days) at 8000 rpm for 20 min to remove cells. The pH of the culture supernatant was adjusted to 2.00 with 1.N HCL and an equal volume of chloroform methanol was added (2:1) The mixture was shaken well for 1 min in separating funnel and allowed to stand until phase separation takes place. The organic phase was then separated and extraction was repeated twice more. The pooled extract was concentrated in a rotary evaporator at 40^0 c to obtain total biosurfactant which was then dried to constant weight at 50^0c under vacuum (Nasrinsamadi, Neda Abadian, Abbas Akhavan, Mohammed Reza Fazeli, Abbas Tahzibi and Hussein Jamalifar-2007)

4.4 Biochemical analysis:

Thus obtained concentrate of biosurfactant was labeled as C1PS and analyzed by HPLC, FTIR and IR.

4.4.1 Reverse-Phase HPLC analysis.

Reverse phase HPLC analysis of biosurfactant sample C1PS obtained from *Panibacillus sp* (C1PS) was carried out on an Agilent 1100 quaternary HPLC system. The chromatogram of C1PS sample revealed at least 06 peaks, arranged as singlets, doublets and triplet. The detailed Report of HPlC-analysis is as shown after this explanation.

4.4.2 IR-Analysis:-

The IR Spectrum of the biosurfactant sample C1PS obtained from *Paenibacillus sp* in KBr showed strong bands indicating the presence of a peptide component at 3357.32 to 3358.18 Cm^{-1} resulting from N-H stretching mode, at 1643.41 Cm-1 resulting from the stretching mode of the co-N bond, and at 1535.39 Cm^{-1} resulting from the deformation mode of the N-H bond combined with C-N stretching mode. Detailed Report of FTIR (KBr) was showed after this explination.

CHAPTER-V

QUANTATIVE DETECTION OF BIOSURFACTTANT ACTIVITY BY SURFACE TENSION MEASUREMENT BY RISE OF LIQUID IN A CAPLLARY TUBE (CAPLLARY RISE METHOD)

5.1 Introduction

The surface of a liquid acts in many respects in a manner analogous to stretched membrane. The examination of water drop slowly farmed at the end of a glass tube or tap from which it emerges, provides an example of this phenomenon. The water in this case accumulates, as through it were collected in an invisible membrane, until of definite size. When it is detached as a spherical drop.

These effects are due to forces existing in the surface of separation of the liquid from the air or other media in contact with it. This effect is generally known as surface tension.

When the liquid is placed on a horizontal plane surface the form it takes depends for a given liquid, on the material of which the plane surface is made. Thus if water is placed on a clean glass surface it spreads over it, where as if glass is greasy the water takes the form of globules.

The angle contained between the plane surface and the liquid surface is different in each case. If we measure this angle in the liquid we have a measure of angle of contact.

There are different methods used for measurement of surface tension of a liquid one of the methods used to measure the surface tension is rise of liquid in capillary tube methods.

5.2 Theory

If a clean, fine-bored Capillary tube is depressed in to a liquid which wets it, and is then clamped vertically, the lower end of the tube being just below the surface of the liquid it will be found that a column of the liquid remains in the tube so that the surface in the latter is a height ‘h’ can above the free surface of the liquid in the vessel which contains it.

Suppose ‘r’ is the radius of the tube and ‘p’ is the density of the liquid. the forces acting on the liquid in the tube are:

1) The weight of the liquid. This is equal to (the volume of the liquid) X gp. Now the volume of the liquid equal to

V = $\pi r^2 h$ + (Volume of meniscus) for a uniform tube. If “r” is small the miniscus is particularly hemispherical, hence

$V = \pi r^2 h + [(\pi r^2) r - 2/3 \pi r^3]$
$= \pi r^2 (h + r/3)$
i.e. downward force is
$= \pi r^2 (h + r/3)$ gp.

2) The upward force due to surface tension. The line of contact is the intersection of the glass wall and the liquid surface, i.e. a circle of radius r. If α is the angle of contact the upward force, from definition of surface tension, is equal to.

$= 2\pi r T \cos \alpha$

But in the practical cases considered, α=o, Hence for equilibrium, the force (5) & (6) are equal and opposite.

i.e. $2\pi rT = \pi r^2 (h + r/3)$ gp.

and $T = gpr/2 (h + r/3)$

Where,

T = Surface tension

h = height of liquid in capillary

r = radius of capillary bore

p = density of liquid

g = constant having value 980 cm/Sec^2

5.3 Procedure

To find out surface tension T of a liquid medium the glass capillary tube is cleaned thoroughly this may be done by using nitric acid (in which the tube is boiled) and caustic soda the tube is washed in tap water and dined In alcohol and ester, Or the glass tube is allowed to stand for several hours, over night if Possible in a concentrated solution of sulphuric acid (one part) and potassium bichromate (one part).it is then washed in tap water and dried. No need to use distilled water.

The tube is then heated and dram out to a capillary length of uniform bore is chosen and clamped vertically in a beaker in which the liquid whose surface tension is to be determined is kept. Care is taken to avoid. Touching the tube or liquid touching the tube of liquid in this adjustment, for even small trace of grace cause a large variation in the value of surface tension.

The capillary tube now is viewed by means of traveling microscope provided with vertical travels, the lower end of the meniscus is focused and venire reading of microscope is noted. The free surface of the liquid in the containing vessel is next focused.

The tube is viewed by means of a traveling microscope, provided with a vertical traverse, the lower end of the meniscus is focused and the venier reading of the microscope noted. The free surface of the liquid in the containing vessel is next focused. If the liquid surface is just above the top of the vessel, this level may be viewed very readily, and from the venire reading on the microscope scale in this position the value of h may be obtained.

To find r, the tube is broken at the point at which the meniscus rested, and viewed horizontally by the microscope.

By arranging the cross-hairs in the eyepiece to be tangential in turn to the two ends of a diameter, the internal radius may be measured on the venier attached to the traverse.

Alternatively, a weighed amount of mercury of density D may be introduced into the tube and its length observed by means of the microscope, when the tube is horizontally on the bed of the microscope.

Hence r, the mean radius of the tube, may be found. If is the length occupied by the mercury of mass m,

$\pi r^2 lD = m$

The experiment is repeated, using tubes of various diameters and a mean value of T obtained.

An alternative method of measuring h, which also overcomes the difficulty of viewing the liquid through the glass beaker, is one which makes use of a pin bent twice at right angles so that the point is displaced about 1 cm. from its original position. The pin is attached to the capillary tube by means of a rubber band .The point is in this way well removed from the curved surface of the liquid round the tube itself. It is adjusted to coincide with the free surface of the liquid.

The meniscus is first viewed and the reading on the scale of the microscope is noted. The vessel containing the liquid is removed and the microscope is then focused on the pin-point. The difference of the reading obtained gives the value of h. (b) Another way of carrying out the capillary tube method which has some advantages over the former can be made by using the apparatus as used in Jaegers Method. The U-tube and beaker are filled with the liquid under investigation. The capillary tube is selected by a microscope examination so that its end is circular. It is connected to the rest of the apparatus by means of a rubber tube which fits on to the manometer at A. A bent pin is attached to the tube.

This serves to mark the surface of the liquid in the beaker and the distance between the pin-point and the end of the tube, denoted by h, measures the depth of the end below the surface. The pressure within the tube is measured by the difference in levels, H, of the liquid in the limbs of the U-tube. By variation of the level of the water in the flask B_1 the liquid in the capillary tube can be forced down until the level of the meniscus is that at the end of the tube.

This has the advantage that the radius of the tube can be measured at the section where the surface tension acts, and it is not necessary to rely on a

measurement of the average radius as in the last method. The radius should be carefully measured by means of an accurate microscope.

The forces downward are $pgH\pi r^2+1/3\ pg\pi r^3$ and upward $pgh\pi r^2+2\pi rT$ Omitting the effects of atmospheric pressure, this acts equally in both directions.

Thus $T = ½\ pgr\ (H – h + 1/3\ r)$

The term r/3 may be neglected if r is so small with respect to (H – h) that the accuracy is not thereby affected.

It should be noted that a considerable error will arise if H and h do not differ appreciably. This difficulty always occurs when a difference of two measured quantities occurs in a formula.

Table No: 5.3.1

Reduction in surface Tension

Incubation Period (Days)	Initial Surface Tension (dynes/cm)	Surface Tension on incubation (Dynes/cm)	Reduction in Surface Tension (dynes/cm)
4 Days	66.3831	65.9520	0.4311
5 Days	66.3831	65.3018	1.0813
6 Days	66.3831	65.2087	1.1774
7Days	66.3831	65.1932	1.1899
8Days	66.3831	65.1591	1.224

CHAPTER VI

Studies on *Rhizopus nigricans* for biosurfactant production by using plant material

Success of fermentation process depends on both, the choice of correct microorganism and the fermentation medium. The medium should be easily procurable, abundant and economic. Synthetic media, though are essential in preliminary investigations are unsuitable during large scale production due to their prohibitive cost therefore it is necessary to search for a suitable medium obtainable from cheaper natural resources. These materials may either be native in origin or may be obtained as industrial by-products. It was from this point of view 'Deproteinised juice' obtained from plant leaves were used for finding out their ability for the production of 'biosurfactant'.

The deproteinsied Juice (DPJ) is considered as by product of Green Crop Fractionation (GCF) system; which can be used in various ways, though the appropriate utility is not yet established. DPJ is rich in water soluble carbohydrates, free amino acids, minerals, lipids and vitamin. The Dominant carbohydrates in DPJ are glucose and fructose.

However, the content of reducing sugars in DPJ in subject to great change, depending upon species and maturity of the plants used it varies from 0.5 to 6.0 %, the contents of nitrogenous substances also vary widely i.e. the concentration of nitrogen sources is 0.02 to 1%.

6.1 DPJ preparation

	DPJ of plants	Dry weight Mg/100ml	Emulsion Index
A	(Spinach) (6)	146	5.01
B	*Brassica oleracea* (Cabbage) (7)	172	5,14
C	*Coriandrum sativum* (Coriander)	150	3.21
D	*Fenugreek sp.* (Methi)	161	3.10
E	*Zea mays* (Maize)	180	4.5
F	*Sorghum vulgure* (Jowar)	174	4.8
G	*Cajanus cajan* (Tur) (3)	182	14.5
H	*Glycine max* (Soyabean) (2)	271	17.8
I	*Phaseolus radiatus* (Mung)	152	3.54
J	*Raphanus sativum* (Radish)	141	4.09
K	*Daucus carota* (Carrot)	156	3.81
L	*Eucalyptus sp.* (Eucalyptus) (5)	285	24.87
M	*Moringa sp.* (Moringa) (4)	282	18.21
N	*Ricinus communis* (Castor) (1)	271	15.12
	Modified GN medium (control)	320	20.05
	Total	3003	142.61
	Mean	200.2	10.18
	S.D.	59.80	7.78

DPJ was prepared as method described by Davys and Pirie (1969), Davys et *al.*, (1969). DPJ of Spinach, Cabbage, Coriander, Methi, Maize, Jowar, Tur, Soyabean, Mung, Radish, Carrot, Eucalyptus, Moringa and Castor were used.

2.5 grams of powder of DPJ was dissolved in 100ml distilled water, pH was adjusted to 6.5. It was sterilized in autoclave at 121.6^0C for 15 minutes. Sterilized DPJ medium was used for study.

In 250 ml Erlenmeyer flask 100 ml DPJ medium was inoculated with *Rhizopus nigricans* (Previously isolated and tested for production of biosurfactant production in known laboratory medium)and incubated on rotary shaker at 120 rpm at 28^0C for 7 days. Modified GN medium was used as control.

Results shown in table-6.1 indicate that natural medium was found suitable for growth and biosurfactant production. DPJ of Eucalyptus was found conducive for growth and biosurfactant production, hence used in further studies.

Table-6.1 Growth and biosurfactant production of *Rhizopus nigricans* in DPJ medium

6.2 Growth optimization and biosurfactant production by *Rhizopus nigricans* in DPJ medium:

DPJ of Eucalyptus was found most supportive for growth and biosurfactant production by *Rhizopus nigricans*. DPJ Eucalyptus was analyzed for its chemical content. Analysis was carried out in Fertilizer Testing laboratory, Aurangabad (MS). Results are noted in table -6.2

Effect of DPJ concentration, temperature, aeration, incubation period was studied. Results are noted in Table- 6.3 to 6.7.

Quantitative detection of biosurfactant production was determined by measurement of Surface Tension by using Capillary rise Method.

Results are noted in Table- 6.8.

Table- 6.2: Chemical composition of DPJ

Sr. No.	**Parameters**	***Composition (%)***
1	Total Nitrogen	*2.22*
2	Total P2O5	*0.32*
3	Total K2O	*2.38*
4	Sodium	*0.41*
5	Copper	*0.00052*
6	Iron	*0.023*
7	Manganese	*0.022*
8	Zinc	*0.009*
9	*Ash*	*10.80*

Table –6.3: Effect of DPJ concentration on growth and biosurfactant production of *Rhizopus nigricans* in DPJ medium:

DPJ %	**Dry weight Mg/100ml**	**Emulsion Index**
0.5	173	16.78
1.0	257	19.99
1.5	280	22.01
2.0	331	23.63
2.5	381	24.86
30	383	24.52
3.5	387	21.31
4.0	390	18.01
4.5	392	13.33
5.0	395	13.29
5.5	398	10.7

6.0	401	10.1
6.5	404	9.97
7.0	405	9.82
7.5	405	8.01
8.0	399	7.59
8.5	352	7.1
9.0	303	5.87
9.5	271	5.23
10.0	202	5.11
Total	6909	277.23
Mean	345.45	13.86
S.D.	72.64	6.90

Table –6.4: Effect of pH on growth and biosurfactant production of *Rhizopus nigricans* in DPJ medium:

pH	**Dry weight Mg/100ml**	**Emulsion Index**
4.0	243	15.05
4.5	282	19.75
5.0	302	20.92
5.5	322	22.05
6.0	341	23.92
6.5	383	24.87
7.0	347	16.02
7.5	206	10.05
Total	2426	152.63
Mean	303.25	19.08
S.D.	58.05	5.02

Table-6.5: Effect of temperature on growth and biosurfactant production of *Rhizopus nigricans* in DPJ medium:

Temperature (^{0}C)	**Dry weight Mg/100ml**	**Emulsion Index**
25^{0}C	285	22.01
28^{0}C	381	24.85
37^{0}C	302	11.61
40^{0}C	217	8.87
45^{0}C	110	6.61

Total	1295	73.95
Mean	259	14.79
S.D.	101.70	8.15

Table –6.6: **Effect of aeration on growth and biosurfactant production of *Rhizopus nigricans* in DPJ medium:**

Shaking (rpm)	Dry weight Mg/100ml	Emulsion Index
80rpm	258	20.12
100rpm	337	23.90
120rpm	381	24.98
140rpm	342	21.15
160rpm	298	10.21
Total	1616	100.36
Mean	323.2	20.07
S.D.	46.82	5.86

Table –6.7*:* Effect of incubation period on growth and biosurfactant production of *Rhizopus nigricans* in DPJ medium:

Incubation period (days)	Dry weight Mg/100ml	Emulsion Index	Biosurfactant production mg/100ml
2	113	5.21	45.2
3	131	7.34	53.7
4	145	10.03	59.4
5	180	19.87	75.6
6	280	24.82	117.6
7	382	25.02	171.6
8	384	24.91	157.4
9	390	9.75	105.3
10	393	9.40	58.9
11	394	9.11	55.12
12	396	8.02	51.48
13	396	7.71	47.52
14	397	6.01	43.6
15	288	5.02	31.6
Total	4269	172.22	1074.02
Mean	304.93	12.30	76.72
S.D.	114.14	7.71	44.06

Table –6.8: **Quantitative measurement of biosurfactant production by using Capillary rise Method:**

Incubation period (days)	Initial Surface Tension (dynes/cm)	Surface Tension on incubation (dynes/cm)	Reduction in Surface Tension (dynes/cm)
4	6.465	5.714	0.751
5	6.465	5.745	0.720
6	6.465	5.764	0.701
7	6.465	5.775	0.690
8	6.465	5.765	0.700
9	6.465	5.762	0.703
10	6.465	5.762	0.703
11	6.465	5.762	0.703
12	6.465	5.762	0.703

6.3 Laboratory scale studies on biosurfactant production in *Rhizopus nigricans:*

Biosurfactant production in batch fermentation:

The last variant in the present studies was to study the biosurfactant production in *Rhozopus nigricans* on the unit of Biostat B, 5 Lit. Automated fermenter (B.Braun Biotech International, Germany) (Photo plate- 12), using the DPJ medium.

Information about Fermenter-

The batch fermentation was carried out in fermenter. Working volume of three litres of DPJ medium was used. The inoculum was grown at 28^0C for 48 hours with shaking in the same medium that was to be used for fermentation. The level of the inoculum used was 3% (v/v). The fermentation process was carried at 28^0C. The pH of the fermentation medium was maintained at 6.5 with 1N NaoH. The aeration rate was 1v/v per minute and agitation rate was 400rpm. The fermentation was run for eight days.

Results are shown in the table- 6.9.

Table 6.9: Production of biosurfactant in batch fermentation on laboratory scale

Incubation period (days)	Emulsion Index	Biosurfactant produced Mg/100ml
1	4.1	37.2
2	5.43	43.7
3	7.71	52.9
4	9.98	58.9
6	16.87	72.6
6	23.96	109.7
7	24.92	159.6
8	23.06	159.1

(Dry weight on 8 days of incubation was measured as 378 mg/100ml)

12. Automated fermenter (B.Braun Biotech International, Germany)

Result and Discussion

After deciding optimum conditions for growth and biosurfactant production in the selected isolates, Deproteinised Juice (DPJ) of plant materials were selected for the production of biosurfactant. Since *Rhizopus nigricans* showed optimum growth and maximum biosurfactant production. It was selected for further studies. DPJ from different plants were used as substrate for production of biosurfactant by *Rhizopus nigricans.*

Surface-active compounds commonly used in industries are chemically synthesized. However, biosurfactants have been paid increasing attention to replace synthetic surfactants due to their advantages such as biodegradability and low

toxicity. Nowadays, the use of biosurfactant has been limited due to the high production cost (Suppasil Mineral, 2004). Microorganisms used various renewable sources, especially agro industrial wastes as potential carbon sources. Olive oil, mill effluent, animal fat, frying oil, babassu oil, molasses, whey, starch rich wastes, were used for biosurfactant production (Morques, 2001; Desphande and Daniel, 1995; Haba et *al*., 1999; Christen et *al*., 2000; M.H. Vance Harrop et *al*., 2003). These low economic cost value materials directly affect the cost of biosurfactant production and reduced pollution caused by those wastes.

Deproteinised leaf juice (DPJ) was used for growth, biomass and alcohol production (A. M. Mungikar and D.P. Geogle, 2000; D.A. Doiphode, 2005).

DPJ of Spinach, Cabbage, Coriander, Methi, Maize, Jowar, Tur, Soyabean, Mung, Radish, Carrot, Eucalyptus, Moringa and Castor plant were used for the production of biosurfactant by *Rhizopus nigricans*. Modified GN- Medium was used as control.

Rhizopus nigricans showed optimum growth 285mg/100 ml and maximum biosurfactant production in DPJ of eucalyptus (Table 6.1).

L. A. Sarubbo et al. 2006 used cottonseed oil for the production of biosurfactant by *Candida glabrata*. The maximum of biosurfactant production was observed by using 7.5% cotton seed oil and 5% glucose, reaching values of 10. g /lit after 144 hours at 200 rpm. The cell free culture broth containing the examined agent lowered the surface tension of the medium to 31 mN/m. The emulsification capacity practically unaltered within a wide pH (2-12), temperature (4^0C-80^0C) ranges and under NaCl concentration upto 10%.

Mabel H. Vance-Harrop et *al*., (2003) reported *Candida lipolytica* produced bioemulsifying agent when grown in the medium containing 1% glucose and

supplemented with 5% babassu oil. Fermentation was run at 28^0C on orbital shaker at 150 rpm for 240 hours the pH was 7.5 at initial stage of fermentation and decreased to 5.6 in the end. The maximum specific growth rate was μ max = 0.024^{-1}. The emulsification activity was 0.666 units at 144 hours of incubation.

In the present study *Rhizopus nigricans* when grown on eucalyptus DPJ medium, with 2.5% DPJ medium, pH 6.5, temperature 28^0C, on orbital shaker at 120 rpm, on seven days of incubation produced 382 mg/100ml of growth (dry weight basis) and 25.02 bioemulsification activity (Table 6.7). Biosurfactant production was noted 171.6 mg/100ml on seventh day of incubation. With respect to dry weight the biosurfactant production is 44.9%. The extra cellular biosurfactant reduced surface tension 0.690 dynes/cm (Table 6.8). However bioemulsification activity noted in GN-medium was 20.05. The activity was found increased in eucalyptus DPJ medium. Zhon and Kosaric, (1995) reported optimal yields of biosurfactant are usually obtained when carbohydrate and vegetable oil are used as substrate. t-value calculated at 5% level is 2.17(Stat analysis -1) for biosurfactant production, which was found significant to carry out biosurfactant production at laboratory scale batch fermentation.

Laboratory scale batch fermentation was run for 8 days. The working volume of DPJ medium used was three liters. On seventh day of incubation biosurfactant production was noted maximum (Table- 6.9).

On seventh day of incubation emulsion index calculated was maximum i. e. 24.92. However it was minimum at 24 hours of incubation. On eight day of incubation emulsion index was found decreased. On seventh day of incubation biosurfactant production was 159.6 mg/100ml. As compared with production in batch fermentation, production at laboratory scale was found decreased by 12.0 mg/100ml. However t-

value calculated at 5% level is 2.44 (Stat analysis- 2) for biosurfactant production which was found to carry out biosurfactant production at laboratory scale fed-batch and continuous fermentation.

CHAPTER –VII

FINDINGS AND CONCLUSIONS

The increased interest in the Study of extermophilic microorganisms is mainly due to the fact that they are considered as an important biotechnological resource and their specific properties are expected to result in novel process applications. Useful insights gained from the study of extremophilic products like (enzymes, proteins, others also)

Alkaliphilic Bacillus/microorganisms include industrially important microorganisms as they produce many extracellular proteins and other products which are stable at extreme pH value i.e. High pH values. Such alkaline proteins and other bacterial products find important use in various field of industry such as leather, detergent, paper, waste water treatment, oil production and also many other industries.

The present study was carried to find out use of alkaliphilic mi - croorganisms for the production of biosurfactants. In this study total 19 alkaliphilic isolates were obtained from a soil and water sample collected from the extreme alkaliphilic environment like lolnar lake, lonar, Dist -Buldhana (Maharashtra). These 19 cultures/ isolate includes

Bacterial 17, fungal cultures 01 and actinomycete culture 01

All these 19 (Table No 3.3) cultures were tested by Lipolytic activity, out of which five bacterial cultures were found to be efficient for lipolytic activity.

These five cultures were tested for biosurfuctant production, from above five only three bacterial isolates named C_1PS, C_1C, and C_3K were found efficient for biosurfactant production (Table No.3.5.1 & fig no 3.5.1)

Of these three bacterial cultures C_1PS was found most efficient for biosurfactant production was identified by 16S rRNA sequencing and found to be *panibacillus spp* chapter III (3.9).This culture grows efficiently at pH -10.7.

Microbial compounds that exhibit decrease in surface tension or showing emulsifying activity are classified as biosurfactants

(L.Rodgrikgues, 2006).

Biosurfactants are structurally diverse group of surface active molecules synthesized by microorganisms (Lu, J.R Zahu, X.B and Yaseen.M, 2007) these are amphipilic compounds produced on living surfaces, mostly on microbial cell surfaces, or excreted extracellularly and contain hydrophobic and hydrophilic moieties that confer the ability to accumulate between fluid phases, thus reducing surface and interfacial tension at the surface and interface respectively. (Karanth N.G.K, Deo.P.G. and Veenanading. N.K, 1999).

Bacteria were greatly explored for the production of biosurfactant and their applications in various fields, Rhamnolipids from *Pseudomonas areuginosa*, surfactin from B. subtilis, emulsion from *Acnetobacter calcoaceticus* and sophrolipids from *Candida bombicola* are some examples of microbial products as biosurfactants.

Initially biosurfactants are used as hydrocarbon dissolving agents in late 1960s; their applications have greatly extended in the past five decades as an improved alternative for chemical surfactants (carboxylates, sulphonates, and sulphate acid esters), especially in food, pharmaceutical and oil industries (Banet, I.M., Makkar, R.S, and Cameotra, S.S., 2000., Desai J.D. and Banet, I.M. 1997). *Bacillus pumilus* (Naruse N.Tenmyo O. et. al 1990) *Bacillus lichniformis* (Jenny, Ketal 1991. Lin S. et al 1994, Yakimov M.*et. al* 1995, Grangemard J, *et. al* 2001)

Growth associated production of biosurfactants by bacteria in which parallel relationships exist between growth, substrate utilization and biosurfactant production. For ex production of rhamnolipids by some *pseudomonas spp*, (Robert, M.M, Mercude, M.P. Bosch. J.L. Parra M.J Espuny, M.A. Manresa, and J.Guinea 1989, Syldatk, C., S. Lang, and F. Wagner 1974, Yamaguchi, M. A. Sato, and A. yakuyama 1976) glycoprotein Ap-6 by *P fluorescens* 378, surface active agent by

B. cereus IAF- 346 and biodispensan by *Bacillus sp* strain IAF-343

(Cooper D.G. and B.G. Goldenberg-1987) also A Calcoaceticus RAG-1 reported to be mixed growth associated and non-growth associated type

(Goldman, S., y. Shabtai, C.Rubinovitz, E Rosenberg and D.l.Gutnick. 1982, Gutnick D.L., E.A Bayer, C.Rubinovitz, O Pinus, Y. Shabtai, S Goldman and E. Rosenberg-1980)

Some bacteria produces biosurfactants under growth limiting conditions viz. *Pseudomonas spp* in nitrogen and iron limited conditions (Guerra-santos, J,H, Kappeli and A Flechter-1984, Mulligan, C.N and B.F. Gibbs-1989, Ramana K.V, and N.G. Karanth-1989) *Candida tropicalis* II P-4 (Singh M.V, Saini, D.K. Adhikari J.D. Desai and V.R. Sista-1990) *Torulopsis apicola* (Hommel R.K. O.Stuwer, W.Stuber, D. Haferburg, and H.P. Kleber-1987).

Bacteria generally exploited for bisurfactant production includes *Rhodococcus erythropolis* (Uchida, Misava S.*et al* -1989) Streptococcus thermopiles (Busscher H. J. Neru T. *et al.* 1994, Rodrlgues L.R. *et al*-2004, Rodrigues L.R. and Banat I.M-2006, Busscher H.J. and van Hrogmoed C.G-1997, Busscher H.G. *et al* 1999) *Streptococcus mitis* (Pratt Terpstra I.H. *et al*-1989, Van Hoogmoed C.G. *et.al*-2000). *Lactobacillus spp* (Velraeds M.*et al*-1997, Reid G. *et al* 1999, Velraeds M. Reid G. *et al* 1996) *Lactobacillus lacties* (Rodrfgues L. *et al* 2004), yeasts- *Candida antartica* (Kitamoto D.*et al* -1993, Isoda Hand Kimoto D. *et al.* 1997, Isoda H and Shinmoto H *et al* 1999, Shibahara M.*et al* 2001), *Candida glabrata* vcp 1002 (L.A. Surubbo *et. al* 2006) *Candida bomicola* (Ito, S and Inoue-1982) fungi have given less attention for biosurfactant production. It is found that large amount of surface active compounds/biosurfactants are produced under nitrogen starvation conditions (Sandra Hewald *et al* 2005 *) Aspergillus fumigatus* was used for biosurfactant production in solid state process using fixed bed column reactor (Vilasia Guimataes *et, al*).

Now a days interest is increased in use of extremophiles for the production of various microbial compounds as they are stable at extreme environments (Bertus van den Burg -2003) Classification of extreme environments refers to the wide variety of different conditions to which microorganisms have adapted. The products including biocatalyst obtained from these microorganisms can be applicable in similarly diverse conditions.

For ex Thermophilic extremophiles have attracted most attention in particular extremophilic proteases, Lipases and Cellucuses, Chitinases and amylases. These thermopiles has growth temperature more that 60-80^{0}c and some are requiring temperature more than 80^{0}c are culled as hyperthermophilie, similarly psychrophyles requiring temperature below 15^{0}C, Halophiles requires salt concentration 2-5 M. NaCl., Alkalophiles requires pH more than 9, Acidophiles requires pH less than 2-3, and Piezophiles requiring pressure up to 130 Mpa. (Bertus van den Burg-current opinion in microbiology. 2003). At present, only a minor fraction of microorganisms on earth have been exploited.

Alkaliphilic bacilli include industrially important species since they can produce many extracellular enzymes which are active and stable at high pH values. These alkaline enzymes including Lipase Find use in various field of industry such as leather, detergent, paper industries and also in waste water treatment. Bakery, Beverages, chemicals, cleaning, cosmetics, Dairy, fat and oil, food processing, meat and fish, in pharmaceuticals for flavors improvement, armo improvement, emulsification.

Also microbial lipases are studied in detail in bacteria like *Chromobacterium viscosum* , *Pseudomonas fluorescence*, yeasts, *candida cylindricae*, *candida currata, candida deformans, Burkholderia glumae strain PGI, Aspergillus flavus* USM A 10 (How shiao Pau and Ibrahim che Omar, Pakistan Journal of Biological sciences-2004) Basidiomycetes like Geotrichum like R-59

Borjana K. Tuleva *et.al* 2002 reported lipase production and emulsification activity.

Till now most of the extremophiles have been studied for various enzyme productions but very less attention is given towards use of alkaliphilic bacteria for the production of biosurfactants or surface active agents. In the present study a sincere attempt has been done for isolation of alkaliphilic bacteria and use of such bacteria for production of alkaline lipase as well as biosurfactants.

In this regard when study is initiated, total 19 cultures Table. No3.3 were studied for maximum lipolytic activity and same lipolytic cultures were used further study like primary screening of biosurfactant producing microorganisms among which six bacterial isolates were

Found to produce lipase Table No.3.5.1

These six lipolytic bacterial cultures were examined for biosurfactant production. Among these isolates named C1PS, C3K , C1C, are found to be efficient biosurfactant producers (Table- 3.7.1).

The yield of biosurfactant was greatly depends on nutritional environment of growing organism (N,G, K Karanth *et .al* 1999)

Among extremophiles different organisms able to grow in extreme environments are studies for various microbial productions like enzymes protease, amylase, xylanase, chitinase, pectinolytic enzymes, Lipase. These enzymes mostly produced by *bacillus Sp.* Other examples of extremophiles includes *B. fibrisolvens*, consists of xylanolytic active proteins(Lin and Thomson, 1991) Thermophilic organism *Thermoanaerobacterium sp,C xylanolytica* xylanse can also be obtained from fungi *cochiobolus carbonum*, *Trichoderma ressei* (Lappalainen. 1986) Recently xylanases from an alkaliphilic *Bacillus sp* Strain K-1 have been Purified (Ratankhanokchai *et. al* 1999), also thermophilic bacteria which produces xylanase includes *Bacillus acidocaldarius* can grow at (figure in bracket indicates optimum temperature and pH respectively) (80^0C,4)*Bacillus stearothermophilus* can grow at (75^0 C6.5)*Bacillus spp* SPS-o at 75^0C,7) *Bacillus amyloliquefaciens* at (80^0 C,7) *Thermophilic bacillus sp* XE(75^0C,6) *Bacilius stearothermophilus* 21 (60^0C,7) *Clostridium stercorarium* (75^0C7,) *Clostridium stercorarium* Hx-1 ($75\ ^0$C 6,5) *Clostridium thermolacticum* TC21 (80^0C , 6.5*) Streplomyces* T7 (Optimum Temperature. 60 ^{0}C , 5.5) *Streptomyces. sp* B-12-2 (65^0C ,6) *Streptomyces thermoviolaceus* OPC 520 (70^0C, 7), *Thermonospora Curvata* (75^0C ,7.8) *Thermotoga sp.* Fj 553.31 (105^0C, 5.3) *Thermotoga maritime* MSB 8 (92^0C,6.2), *Thermotoga thermarum* (100^0C -7, 80^0C, 6)

Ex. of alkaliphilic bacteria used in production of xylanase are *Alkaliphilic Bacillus* 41m-1 grow at pH and temp. (50^0C -9), *Bacillus sp*-c-125 (70^0C -6-10) *Bacillus sp* TAR-1 (70^0C),*Thermoanaerobacterium sp*. (80^0C, 6-6.2) (shao *et. al*. 1995), *Thermotoga thermarum* (80^0C -6,100 ^{0}C -7) (Sunna and Antranikjun 1996), *Bacillus sp*. C-59^0C -2 (60 ^{0}C -5.5) (okazaki *et al.* 1984, *Aeromonas sp.* (60^0C -5-7, 50^0C -6-8, 50 ^{0}C 7-8) (ohkushi *et. al* 1985), Alkaliphilic fungus having active at pH6-9 has also been reported by Bansod *et.al* 1993, Recently an alkalitolerant xylanase from *Aspeogillus fischeri* (Chandra and chundra, 1996)

Of all these extremophiles very less efforts have been made for the study of production of Biosurfactant or surface active agents following are the few examples of microorganisms studied for biosurfactant production. Thermophilic facultatively anaerobic bacteria strain B160 was studied for biosurfactant production (Farah Nynizawati Daud, Adibah yahya, Madihah Md.salleh, Norazurah Suhaimi and chan Hai xyan, Malaysia), Two new strains of halo and thermotolerant, fermenting bacteria were isolated and found to be surface active agents producing, so far no *Bacteroides species* is known to be both halo-and thermotolerant, as these isolates, (K.Denger, B. Schink hermanyh (1995)

Thus among a very large number of extremophiles very few as above have been investigated for biosurfactant production It was thus worthwhile to explore this little investigated area hence in the present study three bacterial isolates C1Ps, C3 K and C1C are screened.

The preliminary study of growth and biosurfactant production was carried on surface cultures. The surface culture facilitate the observation of microbial colony regarding colour, size, shape, elevation, margin, during the period for which culture is incubated, It also gives information or details about physical and chemical parameters.

Glucose Nitrate medium (GN) (Shinde S.R, 1982) was used to study growth and biosurfactant production.

The physiological parameters necessary to optimum biosurfactant production was deduced by conducting various experiments with cultures of C1Ps, C3 K and C1C.

All isolates were grew best at 28^0C, pH 10.7, and 2.0% glucose concentration, as a substrate. Maximum growth was observed on 7-8 days of incubation, inorganic nitrogen source, potassium nitrate, was suitable for growth of isolates. Growth of bactenal isolates and biosurfactant production was found to be maximum in suspension culture while No growth response or very low /slow growth was found in static type incubation.

Among the three isolates C1PS showed Optimum growth and emulsification activity at pH 10.7. (Table No3.7.1), Temperature 28^0C / Room temperature (Table No.3.8.2.1)potassium nitrate as a nitrogen source, in the concentration (0.1% w/v) (Table No.3.8.5.1) 7.8 days of incubation period (Table No3.8.3.1) in suspension culture (Table No3.8.7.1)

Lipases are produced by many microorganisms (Kamimura *et. al* 2001, Elibol and Ozer, 2000) and higher eukaryotes. Enzyme producing microorganisms include bacteria (Kulkarni and Gadre, 2002), fungi (fodiloglu and Erkmen, 1999, Shimada *et. al.* 1992), yeast (corzo and Revah, 1999) and *actinomycetes* (Sommer *et. al,* 1997) Bertolini *et. al* 1994, Jacobben and Poulsen, 1995, Macedo *et. al*, 1997 reported that lipase from *Geotnchum candidum* (Basidiomycete) is currently the subject of intense investigation and industrial interest. Erlik A. Snellman *et. al* 2002 has reported *Acinetobacter sp* RAG-1 produced extracellular lipase (Lip-A). The same organism was also characterized with respect to its production ability of biasurfactant/ emulsifying agent (Gutnick D.L *et. al* 1991, Rosenberg *et. al* 1979).

In the present study lipase producing alkaliphilic bacteria C1PS, C 3k and C1C were found to produce biosurfactant or surface active agents.

Studies by Rambeloarisoa *et. al* 1984 showed that hydrocarbons and hydrocarbonocllastic bacteria accumulated in the foams i.e. 10^7 to 10^8 bacteria ml^{-1} The ability of bacteria growing on hydrocarbon substrates to produce emulsifying agents is well known (Rosenberg-1986). Thus, the production of

surface active molecules by bacteria could be an important factor involved in foam formation.

Biosurfactants can also grouped in to two categories named

1) Low-molecular-mass molecules with low surface and interfacial tensions and
2) High-molecular-mass polymers, which binds tightly to surfaces (Rosenberg and Ron-1999), Cohen and Eyerowa, 2007) and Sophorolipids (Davila *et.al* 1997) while food emulsifiers (Sheperd *et. al.* 1995) and biodispersan (Rosenberg, 1993) are high molecular. Mass polymers. Among these Rhamnolipids which belongs to low-molecular mass molecules/ glycolipids which are predominantly produced by *pseudomonas aeruginosa* (Burger *et. al*, 1963, Zhang and miller-1995, and Beal and Betts, 2000). Also Rhamnolipids produced by *pseudomonas geruginosa* have been widely studied and reported as mixture of homologous species RL1 (Rhc10 c10), RL2 (Rhc10), RL-3 (Rh c10 c10), and RL4 (Rh2 c10) (syidatk and Wagner 1987, Lang and wagner-1987, Rahman *et.al* 2002b). Also these surfactants are produced by *Pseudomonas fiuorescens* NCIMB 11712. *Pseudomonas putida* 21 BN was studied by B.K. Tuleva *et. al* 2002, for the production of surfactant which changes surface tension when grown in medium containing 2% Hexadecane and 2% glucose as carbon source. At pH 7.2 A number of carbon substrates have been used in many researches Quality and Quantity of biosurfactant production are affected and influenced by the nature of carbon substrate (Singer 1985, Raza *et. al* 2007). Diesel and crude oil were identified to be good sources of carbon for biosurfactant production (Ilori *et.al*, 2005) other water soluble compounds such as glucose, sucrose and glycerol have been reported to be sources of carbon substrate for biosurfactant production (Desai and Banat, 1997, Rahman *et. al* 2002 a) 19, 18 and 1.8 emulsion index were shown by bacterial isolates C1PS, C3K and C1C respectively during their growth in GN medium with 2% glucose concentration.

Change in composition of medium other than carbon source also affects on production of biosurfactants. It has been shown by different researchers that Nitrogen source also plays a key role in biosurfactant production. *Arthrobacter paraffineus* ATCC 19588 prefers ammonium to Nitrate as a inorganic nitrogen source for product ion of biosurfactants. (Guerra-santos, L.H. *et. al* 1984, Mac. Elwee, C.G. *et. al* 1990, Robert *et. al* an Abu Ruwalda *et.al* 1991), *Pseudomonus strain* 44 T1 and *Rhodococcus strain* ST-5 growing on olive oil and paraffin respectively also prefers nitrate as a source of nitrogen for production of biosurfactant (Robert *et .al* 1989, and Abu-Ruwalda *et. al,* 1991) . Biosurfactant production by *A. paraffineus* is increased by the addition of L. amino acids such as aspartic acid, glutamicacid, asparagines and glycine to the production medium (Duvnjak 2., D.G. Cooper and N. Kosaric. 1983) *P. aeuuginosa* (Ramana K.V. and N.G. Karanth 1989, Suzuki, T.H, Tanaka, and S. Itoh. 1974). C.tropicalis II P-4 Sing M., V. Saini, D.K, Adhikari J.D. Desai, and V.R. Sista. 1990) and Nocardia strain SFC-D (Kosaric, N, H.Y. Choi, and R. Bhaszczyk. 1990) Shows biosurfactant production increase in nitrogen limitation. In the Present study different nitrogen sources have been studied for biosurfactant production among these potassium nitrate was found to be the most preferred nitrogen source by bacterial isolates C1PS, C1C and C3K for biosurfactant production.

Like any other chemical reaction production of biosurfactant was affected by environmental factors such as temperature, pH (Rahman *et. al.* 2002 b, Ilori *et.al* 2005, Raza *et.al.* 2007) also production of biosurfactant was affected by agitation and availability of O_2 through their effect on cellular growth and activity (Desai and Banat 1997). In *Pseudomonas .sp* biosurfactant production was found maximum at a pH range from 6 to 6.5 while its production decreases sharply above pH-7. (Guerra, santos *et. al* 1984). While Powella *et. al.* 1989 reported pH range 6.5 to 8 shows no effect on biosurfactant production in *Nocardia Corynebacteriodes*. In the present study as alkaliphilic bacteria are used for biosurfactant production all bacterial isolate Shows biosurfactant production at pH 10.7 only Among bacterial isolates,

C1PS showed maximum biosurfactant activity and production at pH 10.7 while other isolates does not show biosurfactant production below pH 10.7.

Effect of Temperature on biosurfactant production by *Arthobactor parafineus* and *pseudomonas sp.* Strain DSMC 874 was reported by Drawin and cooper 1992, Syldatk C.S. *et.al* 1985) Temperature has direct influence on biosurfactant production Banat J.M. 1993 reported a *Thermophilic bacillus*. *sp* that grew and produced biosurfactant at temperatures above 40^0C. or up to 50^0C. Synthesis of biosurfactant by microorganisms has been studied mainly in mesophilic environments using mesophilic organisms Biosurfactant produced from *pseudomonas aeruginosa strain* P-CG3 has effectively enhanced at 50^0C c (Wong j.w.c, Fang M,Zaho Z, and Xing B-2005) In the present study bacterial isolate C1PS *paenibacillus Spp* (C1Ps) showed maximum biosurfactart production at room temperature/28^0C.

Robert *et.al* 1989 and Abu- Ruwalda *et.al*-1991 reported *Pseudomonas strain* 44T1 and *Rhodococeus strain* ST-5 growing on Olive oil and paraffin, respectively, started production after 30 hrs of growth, when culture becomes nitrogen limited up to 58 hrs. Continue to increase biosurfuctant production L. Rodrlyues *et. al* 2006 reported biosurfactant production is optimum at 72. hr of incubation when strains of *Lactobacillus spp* were in stationary growth phase. In the present study *Paenibacillus spp* (C1PS) showed maximum biosurfactant production after 7 to 8^{th} days of incubation while after 9^{th} day of incubation biosurfactant production by *paenibacillus sp* (C1PS) was decreased.

During study on biosurfactant production Nasrin samadi, Neda Abadian, Abbas Akhavan, Mohammad Raza fazeli, Abbas Tahzib and Hussein Jamalifar Tehran, Iran-2007 Reported *Brevibacterium sp*. Strain S-34 showed maximum biosurfactant production at 200 rpm after seven days incubation on rotary flask shaker, (H.Rashedi, E Jamshidi M. Mazaheri Assadi and B. Bonakdaopour-2007) Reported that strain *Pseudomonas aeruginosa* isolated from oil was produced biosurfactant at 120 rpm incubated on rotary flask shaker at 30^0C after 7 days. (Fareh Nunizawati Daud, Adibha yahya, Madihah Md. Salleh, Norazurah Suhaimi and chan Hui Xuan Malaysia) reported locally isolated

thermophilic facultatively anaerobic bacteria strain B 160 shows optimum BS production at 150 rpm at temperature varied between 30 c-70^{0}C. Spencer J.F.T, 1979 reported BS production increases when agitation and aeration rates increased in yeasts. (Shappard and cooper 1990) have concluded that O_2 transfer is important parameter in process optimization for BS production in *B.Subtilis.* In the present study, in suspension culture or culture of C1PS, C1Cand C3K incubated on rotary flask shaker showed maximum biosurfactant production while the same culture at static incubation does not showed biosurfactant production.

After optimization of growth and biosurfactant production conditions for three bacterial isolates, one most efficient culture i.e. C1PS was identified and found to be *paenibacillus sp.* was used for further study for biosurfactant production. This culture was a Gram negative, Rod shaped, catalase positive having other cultural and biochemical characteristics as in Table No3.6.3.1& 3.6.4.1.

After deciding optimum conditions for growth and biosurfatant production for selected three bacterial isolates, Bacterial isolate C1PS was found to be most efficient for bisurfuctant production which was used further for biosurfactant production using the modified GN medium of pH-10.7 at optimized conditions as a substrate.

Surface active agents (BS) which are commonly used in Industries are chemically synthesized. Now days a number of biasurfactants have been researched. The usefulness of biosurfactants (BS) to man in most aspects of humen life can not be over emphasized. The enormous market demand for surface active agents is currently met numerous synthetic, petroleum based chemical surfactants. But these chemically synthesized compounds are toxic to environment and non-biodegradable. (Pattana-thu K.S.M. and Edward Gakpe, U.K-2008). Now a day, the use of biosurfactant has been limited due to high production cost. (Suppasil Mineral, 2004). It has become necessary that tightening environmental regulations and increasing awareness for the need to protect ecosystem have increased interest in biosurfactant as possible alternates

to chemical surfactants. (Banat *et. al.*2000, Benincasa, 2007) Microorganisms used various renewable sources, especially agro industrial wastes as potential carbon sources, especially agro industrial wastes as a potential carbon source, olive oil mill effulent, animal fat, frying oil, babassu oil, molasses, whey, starch rich wastes were used for biosurfactant production. (Morques 2001, Deshpande and Daniel 1995, Heba *et.al,* 1999, Christen *et al,* 2000, M.H. Vance Harrop *et .al,* 2003) These low economic cast value materials directly. Affect the cost of Biosurfactant production and reduces the pollution caused by them in the environment.

During past few decades it has become clear that microbial communities can be found in the most diverse conditions, these extremophiles produces biocatalysts that are functional under extreme conditions. These exteremophiles have a great economic potential in many industrial processes including agricultural, chemical and pharmaceutical applications. Many consumer products will increasingly benefits from exploitation of extremophiles. (Bertus van den Burg, current opinion in microbiology, 2003)

A variety of microorganisms inhabit extreme environments. Extreme is a relative term, which is viewed, compared to what is normal for humen beings for ex low temperature (cold) environment prevail in fresh and marine water. Ocean represents 71% of earth's surface and 90% by volume, which is at 5^0C or colder. Though high Temperatures are not as widespread as cold temperature. A variety of high temperature, natural and man maid habitats exists. The best known and well studied geothermal areas are in north America (yellow stone National Park), Iceland, New Zealand, Japan, Hot water springs are found in India (Manikaran, Himachal Pradesh) In alkaline environments such as soils, increase in pH due to ammonification and sulphate reduction. The best studied and most stable alkaline environments are soda lakes and soda deserts (Ex. East African Rift valley, Indian Sambhar Lake).

Extermophiles from such environments are isolated and are exploited used in production of various biocatalysts, the alkalophilic thermophilic *Bacillus sp.* (NCIM 59) produces cellulose free xylanase at 50^0C and pH-10.0

Five strains of fermenting bacteria were enriched and isolated from oily sludge under conditions of enhanced salt concentration (approx 8% w/v) and temperature 50^0C . These isolates were studied for emulsifier or biosurfactant production of which two strains Glc 12 and strain Fru 22 are able to reduce surface tension 40.3 mNm^{-1} and 42.3 mNm^{-1} respectively with 10 mM sugar substrate.

An effective aerobically produced surfactant can lower the surface tension from 72 mNm^{-1} to about 30 m N m^{-1} (Cooper and zajic, 1980), where the surface active substance produced during anaerobic growth of *Clostridium pasteurianum* only led to decrease the surface tension of 55 mNm^{-1} (Cooper *et.al* 1980).

Use of alkaliphilic bacteria for production of first alkaline amylase in Horikoshi-II medium by *Bacillus sp.* Strain A-40.2, (Horikoshi K 1971, production of alkaline enzymes by alkaliphilic microorganisms. Horikoshi K 1971, alkaline amylase produced by *Bacillus* No A-40.2 Agric. Biol chem. 35, 1783-1791, 1971. Horikashi discovered many alkaliphilic *Bacillus strains* producing CGTase (cyclomaltodextrin glucanotransferases). The crude enzyme from *Bacillus sp* 38-2, was a mixture of three enzymes, (Nakamura N and K. Horikashi 1976 characterization of acid cyclodextgrin glycosyl-transferase of alkaliphilic *Bacillus sp* Agric. Biol chem.40:1647-1648. Nakamura N and K. Horikashi 1976.Characterization of some culture conditions of a cyclodxtrin glycosyltransferase producing alkaliphilic *Bacillus sp* Agric. Biol, chem. 40: 753-757.)

In the present study modified GN medium was used as production medium for *paenibacillus sp* (C1PS) which showed optimum growth 200 mg/100 ml and maximum biosurfactant production in the form of Emulsion index (Table No. 4.1).

I.A Surubbo *et.al.* 2006 used cotton seed oil for production of bioemulsifier by *candida glabrata*. The maximum emulsifier production was observed by using 7.5% cottonseed oil and 5% glucose at 200 rpm after 144 Hrs. The cell free broth examined for surface tension reduction found to 31 m

N m^{-1}. The emulsification activity particularly unaltered within a wide pH range (2-12), temperature 4^0C - 80^0C and NaCl concentration 10%

Mabel H. Vance-Harrop *et.al* 2003 reported *Candida lipolytica* produced bioemulsifying agent when grown in the medium containing 1% glucose and supplemented with 5% babassu oil. Fermentation was run at 28^0C on orbital shaker at 150 rpm for 240 Hrs. The pH was 7.5 at initial stage of fermentation and decreased to 5.6 in the end. The emulsification activity was 0.666 units at 144 Hrs of incubation.

(Nasrin Samadi, Nada Abadian, Albas Akhavan, Mohammad Reza Fazeli, Abbas Tahzibi and Hossein Jamalifar, 2007)reported *Brevibacterium sp.* Strain 5-34 isolated from oil contaminated soil. In Babol, north of Iran when grown in liquid mineral salt medium supplemented with 5% canola oil and incubated on rotary shaker at 30^0C and 200 rpm for 3 days. After centrifugation supernatant was collected and surface tension was measured.

In the present study *Paenibacillus sp* was grown in modified GN medium with 2% Glucose, pH adjusted to 10.7, temperature 28^0C on orbital shaker at 120 rpm, After 7 days incubation produced 200 mg/100 ml of growth (dry weight bases) and 20.0 bioemulsion index (Table 4.1). The extracellular biosurfactant reduced surface tension 1.189 dynes/cm (TableNo-5.3.1, Surface Tension)

Biosynthesis of biosurfactants from variety a bacteria and yeasts have been reported (Davila *et. al* 1992, Zhon and Kosaric, 1995, Daniel *et.al* 1999, Lang and Wullbrandt, 1999). Most commonly involving rhamnolipids, trehalose and sophorose lipids. These usually contains various hydroxyl fatly acids and carbohydrates and are characterized by unique surfactant properties (Ron and Rosenberg-2001).

Characterization of biosurfactant produced by*B.lichniformis* BA 550 by KBr, HPLC showed such biosurfactant lichenysin A are less polar. (Michail M. yakimolv, Kenneth N, Timmis, Victor Wary and Herbert L. Frederickson-Applied and environmental microbiology- May 1995)

In the present study biosurfactant produced by *paenibacillus sp*. (C1PS) was characterized by Reverse Phase HPLC analysis of biosurfactant sample C1PS, was carried out on an Agilent 1100 quaternary HPLC system. The chromatogram of C1 PS sample revealed at least 06 peaks, arranged as singlets, doublets and triplet. The detailed Report of HPLC-analysis is as shown in chapter IV 4.4.1and4.4.2

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