REVIEW OF LITERATURE

2.1. An Enterobacteriaceae member - Serratia marcescens

Bacteria are ubiquitous in nature and constitute a large domain of prokaryotic microorganisms. These organisms have been identified and classified into many groups based on their biochemical, morphological and physiological characteristics. *Serratia marcescens* is a Gram-negative, facultative anaerobic rod belonging to the family Enterobacteriaceae. It is often associated with a characteristic red pigment which has made it a part of history and religious folklore for appearing as blood (Patton *et al.*, 2000).

While this organism was known formerly by a variety of names, including *Chromobacterium prodigiosum*, Gaughran *et al.*, 1968 used the name *S. marcescens* that had been assigned by Bizio in 1823. *S. marcescens* was considered originally to be an innocuous, non-pathogenic saprophytic water organism and was often used as a biological marker because of its easily recognised red colonies (Hejazi and Falkiner, 1997). *Serratia marcescens* is supposedly the only pathogenic species in its genus inhabiting a wide variety of ecological niches and responsible for causing several diseases in plants, vertebrates as well as invertebrate hosts (Coulthurst *et al.*, 2006). It has been identified as a nosocomial pathogen in the last two decades and has been associated with urinary and respiratory tract infections, endocarditis, osteomyelitis, septicemia, meningitis, wound and ocular infections (Yah *et al.*, 2007).

In spite of its emergence as a nosocomial pathogen in the recent past, the organism secretes a wide variety of extracellular products like chitinase, several proteases, nuclease, lipase and a biosurfactant. In addition to these, it also produces secondary metabolite in the form of a red pigment called Prodigiosin which has many promising therapeutic attributes (Hejazi and Falkiner, 1997). Pigment production takes place under specific physical and biological circumstances the alteration of which could lead to increased or decreased pigment formation.

2.2. Ecology

There have been several reports of human infections caused by *Serratia marcescens* in recent years. Immune compromised patients are the most common
targets of infection caused by *Serratia*. These include respiratory and urinary sepsis, infection of the meninges and wound. The infections also appear associated with manipulations of the urogenital tract, respiratory ventilation and previous exposure to broad spectrum antimicrobial agents which are largely inactive against this organism. Some strains become endemic in hospitals and the urinary tract of both symptomatic and asymptomatic patients probably serves as a reservoir for nosocomial infection. Common-source outbreaks of sepsis due to *S. marcescens* arise because of the persistence and multiplication of the organism in equipment, such as respirators (Holmes *et al.*, 1990). Catheter-related bacteremia is also commonly associated with *Serratia* infection (Sekiguchi *et al.*, 2004).

In addition to this, *Serratia marcescens* occurs in soil, water, plant surfaces and other environmental sites as well as digestive tracts of rodents and insects (Thangaraj *et al.*, 2011). *S. marcescens* is the only pathogenic species that has been reported to be pathogenic from its genera although rare reports of infection with *S. plymuthica, liquefaciens, rubidaea* and *odifera* exist. Since the early 1900’s, physicians have used *S. marcescens* to study transmission of microorganisms, as it was assumed to be a harmless saprophyte. In the hospital, Serratia tends to colonize the respiratory and urinary tracts of adults, rather than the GI tract. Serratia sp is responsible for 1.4% of nosocomial septicemia. It is also responsible for 2% of lower respiratory, urinary tract and surgical wound infections (Khanfari *et al.*, 2006).

Due to rapid urbanization, waste disposable has become a major issue not only from chemical and agricultural industries but households as well. The cumulative effect of these activities severely contaminates ground water which is a common source of drinking water. Microbial contamination of these water sources subsequently serves as reservoirs of proliferation of many pathogenic microorganisms which can cause deleterious effects on the health of mankind due to consumption of contaminated water (Golas *et al.*, 2002).

### 2.3. Antibiotic Susceptibility

Gram negative bacilli are a common cause of sepsis, pneumonia, surgical wound infections, urinary tract infections and ventilator associated infections in patients admitted to acute care hospitals. Antimicrobial resistance among gram negative bacilli is rapidly on the rise in present times because of indiscriminate use of
drugs and easy availability of such medication over-the-counter in pharmacies. This has become a serious public health concern and a cause for both morbidity and mortality among hospitalized patients (Lockhart et al., 2007).

Infections caused by *S. marcescens* may be difficult to treat because of resistance to a variety of antibiotics, including ampicillin and first and second generation cephalosporins. Aminoglycosides have good activity against the organism but recent strains have developed. The principle working of β-lactam antibiotics is that the killing effect is time dependent i.e. the length of time the bacteria is exposed to the antibiotic concentrations above the MIC which helps to assess a likely clinical outcome. While P-lactamase-mediated carbapenem resistance is rare among the Enterobacteriaceae, it has been described recently for clinical isolates of *S. marcescens* and *Enterobacter cloacae*. This is a significant cause for alarm because carbapenem drugs are supposed to be highly resistant to β-lactamases and overcoming such a strong resistant structure of the drug would mean that the organism is mutating fast to acquire carbapenem resistance (Hejazi and Falkiner, 1997).

Resistances to β-lactams, cephalosporins, and aminoglycosides have been reported, thereby complicating treatment of *S. marcescens* nosocomial infections (Marty et al., 2002). According to Kumar and Worobec (2002), *S. marcescens* infections are difficult to treat because of high resistance to a wide variety of antibiotics including cephalosporins, aztreonam and imipenem. Recently, *S. marcescens* has also been shown to be resistant to fluoroquinolones. Studies have shown that this is due to mutations in the code of important enzymes such as DNA gyrase and/or overexpression of multidrug resistance efflux pumps. Others were of the opinion that *S. marcescens* strains were highly susceptible to imipenem and ciprofloxacin, and intermediate resistance was found to chloramphenicol and trimethoprim-sulfamethoxazole (Loueiro et al., 2002). A recent study conducted showed that there is a synergy between opportunistic pathogens and HIV (Spach and Jackson, 1999). There is evidence to show that the pathogens breakdown the mucosal barrier and permit easier access of HIV to the bloodstream consequently to the T-helper cells and immune cells. The study specifically showed the detrimental association between HIV/AIDS and *S. marcescens* pertaining to urinary and gastrointestinal infections. Due to weakening of the immune system following HIV entry, it facilitated easy thriving of the opportunistic pathogens (Yah et al., 2007).
2.4. Epidemiology

The first description of nosocomial infection caused by *S. marcescens* was Wheat’s report of 11 cases over a 6-month period in 1951 at Stanford University Hospital. Infections caused by this organism have been reported with increasing frequency since 1960. In 1966, McCormack and Kunin reported a nursery epidemic involving 27 babies, although only 15 cases of *Serratia* bacteraemia had been recorded by 1968 (Hejazi and Falkiner, 1997).

Following the above episodes, 1088 *S. marcescens* isolates were identified throughout 13 clinical departments of Pellagrin Hospital (Bordeaux, France) and were studied from 1968 through 1975 (Grimont and Grimont, 1978).

Rastogi et al. (2002) reported the first ever *Serratia marcescens* infected sputum samples in Rajasthan which had not been reported earlier. The frequency and antimicrobial resistance patterns of microorganisms associated with sepsis cases among newborns at a public maternity hospital in Rio de Janeiro city, Brazil during a two year period was reported and *Serratia marcescens* featured as a prominent infectious agent (Loureiro et al., 2002). The incidence of *Serratia marcescens* has also been reported in 2003 in Japan from 23 clinical isolates of *S. marcescens* from 21 inpatients during August and September 2003 (Sekiguchi et al., 2004). An outbreak due to *Serratia* infection was also identified from to 22 February 2001 at the intensive care unit (ICU) of National University hospital, Kuala Lumpur, Malaysia (Alfizah et al., 2004).

Yah et al. (2008) studied the antibiotic resistance pattern of *Serratia marcescens* in HIV infected individuals in Nigeria. Plasmid curing was carried out and the results established that resistance genes were chromosomally mediated and there was the presence of a high antibiotic resistant genes marker among *S. marcescens* strains in HIV/AIDS individuals in Nigeria.

As recent as April 2011, an outbreak of *Serratia marcescens* colonization in the neonatal intensive care unit was seen in Pescara General Hospital in Italy. Rapid investigation revealed the culprit to be the characteristic red pigmented organism which caused sepsis in four surviving infants. Investigative measures
detected the reservoir of outbreak to be two soap dispensers and immediately strict hygiene routines were established (Polilli et al., 2011).

2.5. Pathogenicity and Virulence

*Serratia marcescens* was initially thought to be a harmless, avirulent organism but is now known to cause opportunistic infections of varying type. In addition to the toxic lipopolysaccharide layer in its cell wall, the pathogen also produces toxic enzymes like extracellular proteases which have been reported to cause skin, cornea and lung damage (Lyerly and Kreger, 1979).

According to Holmes et al. (1990), the pathogen is responsible for infections of urogenital tract, respiratory ventilation and previous exposure to broad spectrum antimicrobial agents which are largely inactive against this organism. Some of the *Serratia marcescens* strains have become endemic in hospitals and in the urinary tract of infected patients and carriers hence, serving as a reservoir for nosocomial infections. Persistence of the organism in hospital based equipment and solutions have made the occurrence of septicemia much more frequent. Hejazi and Falkiner (1997), in their review article, have stated that *Serratia marcescens* has been implicated as an aetiological infectious agent responsible for every conceivable kind of infection including respiratory tract infection, urinary tract infection (UTI), septicaemia, meningitis and wound infections. It also causes endemic in communities and hospitals which is due to lapse of hygiene practices during cardiac surgery. In present times, *S.marcescens* has emerged as a complete and recognized pathogen and a rising threat as an infectious agent frequenting healthcare facilities.

Adherence and hydrophobicity:

*S. marcescens* is a cause of nosocomial UTI, possesses pili and adheres to uroepithelial cells. Two classes of adhesins have been suggested. One class, designated mannose-resistant (MR) pili, agglutinates chicken erythrocytes in the presence of D-mannose; the other class, mannose-sensitive (MS) pili, exhibits mannose-sensitive haemagglutination of guinea-pig and chicken erythrocytes. (Yamamoto et al., 1985). Assessment of the effect of bacterial piliation on interaction with human PMNLs was carried out by Mizunoe et al. (1995) who found that *S. marcescens* strain US46, a human urinary tract isolate, seemed to possess both MI2
and MS pili. This finding suggested that MS-pilate bacteria stimulate PMNLs to produce active oxygen radicals, leading to tissue damage of the infected organ.

Lipopolysaccharide:

The lipopolysaccharide (LPS) is present in the outer membrane of all gram negative bacteria which is responsible for its biological activity and high resistance pattern to antibiotics. The O-polysaccharide layer of the LPS contributes essentially to the virulence of the bacterium. According to Oxley et al. (1989), the S. marcescens serotype 016 produces two neutral O-polysaccharides: D-galactan I and a polymer containing 2-substituted p-D-ribo-furanosyl (Ribj) residues. There has been an enormous increase in our knowledge of the fundamental steps in the biosynthesis and assembly of the outer membrane of Gram-negative bacteria. Lipopolysaccharide is a major component of the outer membrane of Gram-negative bacteria as is peptidoglycan. Porins, efflux pumps and other transport proteins of the outer membrane are also present. It is clear that there are numerous essential proteins that have the potential to be targets for novel antimicrobial agents. Progress, however, has been slow. Much of the emphasis has been on cytoplasmic processes that were better understood earlier on, but have the drawback that two penetration barriers, with different permeability properties, have to be crossed. With the increased understanding of the late-stage events occurring in the periplasm, it may be possible to shift focus to these more accessible targets. Nevertheless, getting drugs across the outer membrane will remain a challenge to the ingenuity of the medicinal chemist (Page, 2012).

Most strains of Serratia marcescens secrete a cytotoxin that ruptures or hemolysis human and animal erythrocytes (red blood cells) resulting in the release of inflammatory mediators leukotrienes and histamine from leukocytes. Increased pathogenicity had been observed earlier in E.coli strain 536/21 after transformation with the S. marcescens shLA and shIB genes. The transformed E.coli cells showed the same hemolysis properties towards as the parent S. marcescens strain (Hertle et al., 1999). Multidrug resistance efflux pumps play a major role in the intrinsic and acquired resistance of various human pathogens, with determinants for multidrug efflux pumps being identified in genomes of most, if not all, bacterial species. One group has suggested previously the presence of an efflux mechanism in S.
S. marcescens. The study reported here proves the presence of an efflux mechanism for fluoroquinolones in *S. marcescens* and identifies a resistance–nodulation–cell division (RND) pump encoding gene in this organism that could be a possible candidate responsible for drug efflux (Kumar and Worobec, 2002).

*S. marcescens* was thought to cause infections not only in humans but in plants as well. Cucurbit yellow vine disease (CYVD) first was observed in Oklahoma in 1988 in pumpkin and squash. Exhibiting characteristic symptoms of yellowing foliage, wilting, and phloem discoloration, this disease has since caused severe losses also in cantaloupe and watermelon crops. Disease incidence has varied annually from small, isolated outbreaks to complete crop loss, especially in early-planted fields (8). Originally thought confined to Oklahoma and central Texas, CYVD has since been confirmed in Arkansas (J. C. Correll, personal communication), Tennessee (6), Massachusetts (43), Kansas (N. A. Tisserat, J. Fletcher, and B. D. Bruton, unpublished data), Colorado (B. D. Bruton, unpublished data), and Nebraska (R. M. Harveson and B. D. Bruton, unpublished data) (Rascoe et al., 2003).

Studies of the virulence factors of *S. marcescens* demonstrated that clinical isolates produce a toxin with activity on Vero (African Green monkey kidney) cells in culture. These strains were isolated from distinct clinical sources and no relationship was observed between cytotoxic activity and strain serotype. Recently, it was shown that the hemolysin of *S. marcescens* induces cytotoxic effects on human epithelial cells, characterized by vacuolization with subsequent cell lysis. The cytopathic effects of the *S. marcescens* cytotoxin correspond to cell rounding followed by gradual destruction of the monolayer, as observed by inverted microscopy (Carbonell et al., 2003).

Outbreaks caused by *S. marcescens* strains, are being frequently reported mainly seen as urinary tract infections, wound infections, pneumonia, and bacteremia. Environmental sources of the organism, especially having a predilection for moist conditions, results in an increase in nosocomial infections. Multi-resistance of *S. marcescens* to antibiotics causes a problem in treating infected patients and controlling outbreaks (Alfizah et al., 2004).

Walker et al. (2004) further confirmed the role of the hemolysin produced by *Serratia marcescens* which was encoded by the shlA gene. It was also found to be
cytotoxic to epithelial cells and fibroblasts causing ATP and potassium efflux mechanism depletion. Coulthurst et al. (2006) were of the opinion that S. marcescens strains produce a range of secreted products, including proteases, nuclease, lipase, chitinases and haemolysin, many of which are likely to represent virulence factors in human infection. Khanafari et al. (2006) stated that in the hospital, Serratia tends to colonize the respiratory and urinary tracts of adults, rather than the GI tract. Serratia sp. is responsible for 1.4% of nosocomial septicemia. It is also responsible for 2% of lower respiratory, urinary tract and surgical wound infections. Serratia sp. can be a cause of meningitis, especially after surgical intervention. Also reported is endocarditis and osteomyelitis in heroin addicts.

The mode of transmission of Serratia is mainly via direct contact, droplets, catheters, saline irrigation solutions and in other supposedly sterile solutions. Infections with opportunistic pathogens have been one of the hallmarks of the acquired immunodeficiency syndrome since the beginning of the epidemic. Urinary and gastrointestinal tracts infections account for a sizeable number of these opportunistic infections in hospitalized HIV-infected individuals in developing countries (Yah et al., 2007).

2.6. Biodiversity

Biotyping for Serratia marcescens using eight carbon sources (benzoate, DL-carnutène, m-erythritol, 3-hydroxybenzoate, 4-hydroxybenzoate, lactose, Dqunate, and trigonelline), a tetrathionate reduction test, production of prodigiosin, and horse blood hemolysis was derived from a recent numerical taxonomic study (Grimont et al., J. Gen. Microbiol. 98:39-66, 1977). A total of 98.6% of 2,210 isolates from various sources could be assigned to 1 of 19 biotypes. Distribution and spread of 1,088 S. marcescens isolates throughout 13 clinical departments of Pellegrin Hospital (Bordeaux, France) were studied from 1968 through 1975. Except for one that colonized the intestinal tract of newborns, the six pigmented biotypes were seldom isolated. Each of the 13 nonpigmented biotypes showed a particular pattern of distribution and spread. The usefulness of S. marcescens biotyping was shown by relating several isolates recovered from patients and their inanimate environment and by pointing out the possible existence of infections or colonizations by two unrelated biotypes. S. marcescens strains isolated from the natural environment (water) are
usually pigmented, and their biotypes are uncommon in hospitals (Grimont and Grimont, 1978).

2.7. Genotyping

The polymerase chain reaction (PCR)-based procedures of randomly amplified polymorphic DNA (RAPD) and repetitive element (RE) based PCR were used to amplify total DNA prepared from each of 62 clinical *Serratia marcescens* isolates. Three different random primers, designated 1060, 1254 and 1283, were used individually in RAPD-PCR. Primers representing enterobacterial repetitive intergenic consensus (ERIC) sequences, extragenic palindromic (REP) elements, and polymorphic GC-rich repetitive sequences (PGRS) constituted the repetitive element-PCR. Patton and his co-workers were able to generate 40, 40 and 58 genotypic groupings using the 1060, 1254 and 1283 RAPD primers, respectively. Using the ERIC, REP and PGRS primers, 19, 54 and 60 unique genotypic profiles were yielded, respectively. The PGRS primers, which were developed to amplify GC-rich repetitive sequences in the genome of Mycobacteria, were the most discriminatory. These data indicate that both of these PCR-based approaches are a valid means of discriminating strain differences among isolates of *S. marcescens* and the amount of differentiation depends on the primer used. This is a very useful find in the surveillance and examination of future outbreaks by *S. marcescens* (Patton et al., 2001).

2.8. Numerical Taxonomy

According to the biotyping conducted by Holmes et al. (1990), 18 clinical isolates of *Serratia marcescens* were taken from the two hospitals of incidence and subjected to one dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole cells proteins. The protein patterns were highly reproducible and were used as the basis of a numerical analysis which divided the clinical isolates into eight protein types. Comparison with 0-serotyping indicated that the level of discrimination by SDS-PAGE was similar. As with 0-serotyping, a secondary scheme, such as phage typing was necessary to differentiate strains of the same protein type.
2.9. Molecular Taxonomy

Random Amplification of Polymorphic DNA (RAPD) fingerprinting of three different types of bacteria namely *Pseudomonas aeruginosa*, *Bacillus circulans* and *Serratia marcescens* was carried out using ten different primers. The RAPD-PCR produced reproducible electrophoretic band patterns only in seven primers. There were 67 species specific bands observed with 23 specific bands for *Serratia marcescens*. Based on RAPD data genetic identity between *S. marcescens* and *B. circulans* was more than the other (0.3803) (Thangaraj *et al.*, 2011).

2.10. Phylogenetic Analysis

Homologs of the dimeric HU protein of *Escherichia coli* can be found in every prokaryotic organism that has been analyzed. In this work, *Serratia marcescens* synthesizes two distinct HU subunits, like *E. coli* and *Salmonella typhimurium*, suggesting that the heterodimeric HU protein could be a common feature of enteric bacteria. A phylogenetic analysis of the HU-type proteins (HU and IHF) is presented, and a scheme for the origin of the hup genes and the onset of HU heterodimericity is suggested (Oberto and Yaniv, 1996). There are several genomes that are in the process of being sequenced. Two different strains of *S. marcescens*, ATCC 13880 and Db11, are currently being sequenced, by the University of Wisconsin-Genome Evolution Laboratory and the Sanger Institute, respectively (Mahlen, 2011).

2.11 Industrially Important Products

2.11.1. Prodigiosin – Red pigment

Many microorganisms have the unique property of producing a secondary metabolite which is characterized by a unique color. Other than the fact that these are natural pigments which are safer when compared to their chemical counterparts as an additive especially in the food industry, many of these pigments have unique properties which play an important role in the pharmaceutical industry as well as in being a good source of nutrients such as vitamins and minerals. However, most microbial pigments are water insoluble, have poor stability and show different intensities of color at varying pH, temperature and nutrient sources (Joshi *et al.*, 2003). Nevertheless, microorganisms have the capability of producing a wide variety of pigments such as carotenoids, melanins, flavins, quinines, prodigiosins, monascins.
and violacein or indigo. Fungi and bacteria have the capacity to produce many different colored pigments of varying hues used in the food industry as additives (Venil and Lakshmanaperumalsamy, 2009).

For several decades, prodigiosin has been known to be a natural cytotoxic compound of broad range activity as displayed by Vibrio psychroerythrus, S.marcescens and Pseudomonas magnesiorubra (Furstner, 2003).

2.11.2. Structure of Prodigiosin:

Secondary metabolites of bacterial origin include various enzymes, pigments, and antibiotics which could be of importance to mankind in many ways (Giri et al., 2004). The prodigiosin group of natural products is a family of tripyrrole red pigments that contains a common 4-methoxy, 2-2 bipyrrrole ring system. Its biosynthesis is a two step process in which mono and bipyrrrole precursors are synthesized as two separate entities first and then assembled to form the final product prodigiosin (Figure 1). The pigment is a multifaceted secondary metabolite produced by many microorganisms like Serratia marcescens, Pseudomonas magnesiorubra, Vibrio psychroerythrous, S. rubidaea, Vibrio gazogenes, Alteromonas rubra, Rugamonas rubra and Gram positive actinomycetes, such as Streptoverticillium rubrirecti and Streptomycyes longisporus ruber form prodigiosin and/or derivatives of this molecule. Each of these organisms produces different derivatives of prodigiosins called as prodiginines (Khanafari et al., 2006). Prodigiosin is therefore a typical member of a group of compounds with a common pyrroylpyrro-methene (PPM) skeleton and has a series of close relatives bearing the same PPM core with different alkyl substituents (Wei and Chen, 2005).

The pigment produced by S. marcescens is non-diffusible with a characteristic red color. The non-chromogenic biotypes have been believed to be the opportunistic pathogens posing a public health threat. Chromogenic biotypes have been rarely implicated in causing widespread nosocomial infections (Gargallo and Loren, 1987).
2.11.3. Conditions for pigment expression:

Nutritional components in the growth media as well as physical and biochemical parameters are extremely important for efficient production of prodigiosin pigment with enhanced colour intensity.

Physical factors:

Time:

Cang et al (2000) showed that effective prodigiosin production occurred after 48 hours of incubation and was completed at 72 hours. According to Giri et al. (2002), pigment production is highly variable among species and is dependent on many factors such as species type and incubation time. Nutritional broth containing the isolate were incubated at 24, 30, 36, 48, 72, 78, 84 and 96 hours respectively and prodigiosin production was estimated after incubation. The most effective incubation time was found to be 72 hours (Samrot et al, 2011).

Temperature:

Haddix and Werner (2000) reported in their studies that population growth kinetics of Serratia marcescens are unaltered by a temperature increase to 37°C. The higher temperature evokes a physiological rather than a genetic change to repress prodigiosin expression. In addition to this, pigment expression showed considerable recovery at lower temperatures as the cells reached late logarithmic phase.
According to Hardjito et al. (2002), biomass production of Serratia marcescens did not show any significant change between temperatures 25 and 37°C, however, their optimum temperature was found to be 30°C which also gave the optimum yield of the pigment. At temperature of 30°C, pigment production was greater than that of 25°C. The production of prodigiosin is in S. marcescens is temperature sensitive and the production drastically reduces at temperatures exceeding 37°C (Giri et al., 2004). Tao et al. (2005) showed in their research paper that pigment production was optimum at 28°C.

Studies by Khanafari et al. (2006) further reinstated the earlier research which had suggested that at higher temperatures exceeding 37°C, no prodigiosin was synthesized. This was seen when pigment synthesis was attempted at 39°C and its absence was a clear indication of inhibition of prodigiosin at higher temperatures. Maximum temperature production was seen at 30°C and at pH7 according to Sundaramoorthy et al. (2009).

Samrot et al. (2011) studied the effect of different temperatures on the prodigiosin yield by Serratia marcescens and found the ideal temperature to be less than 30°C, more precisely 28°C which was the same as studies carried out by Tao and his fellow-researchers earlier.

**pH:**

The production of prodigiosin has been shown to be influenced by numerous environmental factors with pH being an important physiological parameter (Venil and Lakshmanaperumalsamy, 2009). This is a very important physiological factor for microbial pigment production because many natural pigments are water insoluble and show changes under different pH conditions (Joshi et al., 2003).

In the year 2000, ethanol was used as a constituent in the production medium of prodigiosin and the pH was maintained at 7.8 after autoclaving the medium. This was determined to be the ideal medium for the optimization of prodigiosin (Cang et al., 2000). In a paper published soon after, the optimum temperature of growth was 30°C and the S. marcescens strain did not grow at pH 4.0, but did grow at pH 5.0 to 11.0. Pigment was produced at pH values of 5.0 to 9.0. At the lower pH, pigment was
produced at 18 hours, while at pH 7.0-9.0, its production occurred at 12 hours concluding that a pH towards neutral and slightly alkaline was preferred for pigment enhancement (Hardjito et al., 2002).

The reduction in prodigiosin production by *S. marcescens* mediated by glucose and other metabolizable sugar was due to a decrease in pH observed in the cell suspensions (Khanafari et al., 2006). The lower the pH, the more acidic the medium and hence, lower the yield of the pigment. According to Mekhael and Yousif (2008), the ideal pH for optimum prodigiosin production was found to be 8. The influence of initial pH of the growth medium on prodigiosin production was investigated by Sundaramoorthy et al. (2009). The prodigiosin production was substantial at pH 7.0. This observation was further justified in 2011 when pH 7 was established as the ideal pH for pigment production in the research work carried out by Samrot et al (2011).

**Nutritional requirements:**

Carbon and nitrogen sources are not only crucial components for the growth and metabolism of microorganisms but also important constituents in the production of bio-enzymes and secondary metabolites which have wide applications in various industries. Prodigiosin is a secondary metabolite produced in the latter part of the growth cycle of *Serratia marcescens* i.e. towards the late log or early stationary phase. As such, secondary metabolites do not play a role in growth, development, and reproduction like primary metabolites do. Hence, nutrients in the form of carbon and nitrogen sources must be such that they do not inhibit the production of the pigment, a secondary metabolite produced in the latter part of the growth cycle.

Many different types of selective and differential media are present that play an important role in the enhancement of the pigment. Capryllate thallous [CT] agar contains caprylate as a carbon source for *Serratia* and thallous salts as inhibitors for other organisms Starr et al. (1976). However, the most common media being used for prodigiosin production has been nutrient broth according to Haddix and Werner (2000), peptone glycerol broth as used in research by Venil and Lakshmanaperumalsamy (2009) and Luria Bertani broth according to Hardjito et al. (2002).
Tao et al. (2005), used a two step feeding strategy using glucose for growth and then glycerol for prodigiosin synthesis, a \textit{S. marcescens} mutant produced about 583 mg prodigiosin/L in 30 h, with glycerol as the sole carbon source in a 5-l bioreactor. This study demonstrated that glycerol allowed the highest prodigiosin production rate when used as main carbon source in the medium among several others carbon sources.

Nakamura and his colleagues used sodium oleate 2\% in the medium and also studied oleic acid substitution instead of sodium oleate and had used only triolein as substrate and reported a yield of 0.69 mg/ml prodigiosin (Nakamura et al., 1986). Giri et al. (2004) reported that the initial comparative work was done using powdered sesame seed in water, nutrient broth and peptone glycerol broth as a growth medium for \textit{S. marcescens}. After having observed sesame seed to give a better yield in terms of prodigiosin biosynthesis further comparison was done with readily available cheaper sources like peanut and coconut. Sesame oil, peanut oil and coconut oil were also compared with the rest of the media. The media were also compared for growth at three different temperatures in terms of prodigiosin production. This led to the observation that fatty acids play a role as the substrate for enhanced prodigiosin production. The various components in the seeds as substrate could have stimulated cell density which in turn could have resulted in higher accumulation of the positive regulator inside the cell thereby triggering excessive pigment production. The powdered peanut seed medium supported the prodigiosin biosynthesis even at 37°C which was not so in the case of nutrient or peptone glycerol broth with and without sugars. The crushed sesame seed broth gave the maximum yield of prodigiosin at 28°C, 30°C and 37°C when compared to nutrient broth and peptone glycerol broth. The maximum prodigiosin production was seen at 28°C and 30°C in nutrient broth. At 37°C \textit{S. marcescens} did not show any pigment production in nutrient broth and the culture broth was white in color. In case of the powdered peanut broth, even at 37°C, pigment production was observed and in fact it was equal to the amount of pigment production seen in nutrient broth at 30°C.

Nutrient broth with glucose showed a two fold increase of pigment at 28°C. The pigment production was more in sesame seed broth even without the addition of any sugars, when compared to sesame seed broth with glucose or maltose. The pigment production was reduced in sesame seed medium with maltose at 28°C when
compared to powdered sesame seed broth exclusively. Glucose in powdered sesame seed medium showed complete decrease of prodigiosin production at both 28°C and 30°C respectively. Amongst the two sugars substituted, maltose acts as a better source of substrate in enhancing pigment production in nutrient broth. This clearly showed that in sesame medium the addition of maltose or glucose does not significantly enhance the pigment production. In fact the addition of glucose or maltose caused a reduction in prodigiosin production which could be due to catabolite repression. The pigment production in nutrient broth with sugars was not more than what was observed in sesame seed medium. Sesame oil broth, peanut oil broth and coconut oil broth as substrate were more efficient in inducing pigment production of 0.76, 2.89 and 1.42 mg/ml respectively when compared to the use of nutrient broth (0.52 mg/ml) or peptone glycerol broth (0.302 mg /ml).

The reduction in prodigiosin production by Serratia marcescens mediated by glucose and other metabolizable sugar was due to a decrease in pH observed in the cell suspensions. Taking into consideration the basic role of carbon source in enhancing pigment production, two justifications can be made. The first point is that in nutrient broth, which is basically devoid of carbon source, the addition of maltose or glucose enhanced the pigment production but not so in the case of sesame broth which already has carbon in the form of fatty acids. The decrease in prodigiosin production seen in powdered sesame seed broth with the addition of glucose or maltose could be due to a catabolite repression. Maltose and glucose added in nutrient broth gave a two fold increase in yield over nutrient broth or peptone glycerol broth alone. The second point is that a slight enhanced pigment production was seen in the case of peptone glycerol broth at 30°C over nutrient broth at 28°C and this could be attributed to the glycerol present as carbon source.

Cang et al. (2000) showed that a strain Serratia marcescens S389, isolated as an ethanol-utilizing bacterium, produced prodigiosin at up to 3 mg ml/1 when grown on ethanol and with the omission of inorganic phosphate and NaCl from the medium. This yield was some 200-fold greater than that previously reported.

Pigment inhibitors:

According to Hejazi and Falkiner (1997), visible light (2000 lux) influenced pigmentation without changing the growth characteristics of the culture. The
maximum prodigiosin content in dark and light cultures was observed on days 3-4 and 2-3, respectively. The influence of illumination on pigmentation of S. marcescens was also demonstrated. Light affects the pigment synthesised by the culture directly. The investigation supported the conclusion that strains of S. marcescens differ in their sensitivity to the effect of NaCl on prodigiosin biosynthesis. Although proline was present in the medium, S. marcescens grown on mineral media did not produce pigment when the carbon source was glucose or the nitrogen source was ammonium chloride.

Giri et al. (2004) reported that addition of maltose to nutrient broth enhanced pigment production only by 2 fold as at 28°C and 30°C. Nutrient broth with glucose showed a two fold increase at 28°C. The pigment production was more in sesame seed broth even without the addition of any sugars, when compared to sesame seed broth with glucose or maltose. The pigment production was reduced in sesame seed medium with maltose at 28°C when compared to only powdered sesame seed broth. Glucose in powdered sesame seed medium showed a complete decrease of prodigiosin production at both 28°C and 30°C. Amongst the two sugars substituted, maltose acts as a better source of substrate in enhancing pigment production in nutrient broth. This clearly showed that in sesame medium the addition of maltose or glucose does not significantly enhance the pigment production. In fact the addition of glucose or maltose caused a reduction in prodigiosin production which could be due to catabolite repression. The pigment production in nutrient broth with sugars was not more than what was observed in sesame seed medium.

The effect of glucose was earlier established in 1976 when glucose was found to cause severe repression of prodigiosin production in Serratia marcescens and a dose related partial reversal was demonstrated by theophylline. It is suggested that this reversal is due to the inhibition of cAMP phosphodiesterase and the concomitant increase in cellular cAMP concentration (Clements-Jewery, 1976).

In their paper, Witney et al. (1977) studied the synthesis of prodigiosin by non-proliferating cells of Serratia marcescens was examined in the presence of a wide range of concentrations of inorganic phosphate (Pi). A high elevation of pigment formation was obtained at -0.3 mM, and a broader but much lower elevation was obtained at 10 to 250 mM Pi. The synthesis of two immediate precursors of the
pigment also was inhibited by Pi. The mechanism of action of Pi did not involve changes in pH or accumulation of the trace metal nutrient iron or zinc. Inhibition was most pronounced when Pi was added to the induction system before the onset of pigment formation. The inhibitor also diminished the burst of alkaline phosphatase activity that occurred in the period between the start of induction and appearance of prodigiosin.

**Haddix et al. (2008)** presented a kinetic model that related cell, ATP, and prodigiosin concentration changes for *S. marcescens* during cultivation in batch culture. Cells were grown in a variety of complex broth media at temperatures which either promoted or essentially prevented pigmentation. High growth rates were accompanied by large decreases in cellular prodigiosin concentration; low growth rates were associated with rapid pigmentation. Prodigiosin was induced most strongly during limited growth as the population transitioned to stationary phase, suggesting a negative effect of this pigment on biomass production. Mathematically, the combined rate of formation of biomass and bioenergy (as ATP) was shown to be equivalent to the rate of prodigiosin production. Studies with cyanide inhibition of both oxidative phosphorylation and pigment production indicated that rates of biomass and net ATP synthesis were actually higher in the presence of cyanide, further suggesting a negative regulatory role for prodigiosin in cell and energy production under aerobic growth conditions.

### 2.11.4. Isolation and Purification of Prodigiosin:

Various isolation procedures for prodigiosin production have been followed by several researchers in an attempt to find the best possible culture medium for maximum production of the pigment. **Cang et al. (2000)** used an ethanol utilizing strain of *Serratia marcescens* which was cultivated using twenty 500 ml Sakaguchi flasks, each containing 50 ml a medium constituting of 15 ml ethanol, 15 g cottonseed meal, 0.5 g MgSO$_4$.7H$_2$O, and 1 ml mineral solution (0.3 g each of CuSO$_4$.7H$_2$O, ZnSO$_4$.7H$_2$O, CoSO$_4$.7H$_2$O, FeSO$_4$.7H$_2$O, and MnSO$_4$.4H$_2$O in 100 ml distilled water, pH 2.3) in 1000ml of tap water, pH 6.8 for 72 h. The culture broth was centrifuged and the products were extracted from the cell pellets with 300 ml acetone. The red supernatant was evaporated in vacuum to a small volume and the concentrate was extracted with chloroform after being adjusted to pH 3.0 with 1 M HCl. The
extract was evaporated to dryness to yield a crude preparation (2.45 g), which was purified by column chromatography on a silica gel using a solvent of toluene/ethyl acetate (9:1, by vol.). Ten ml fractions were collected and examined for prodigiosin by TLC. Fractions having a single prodigiosin spot were pooled and the solvent was evaporated. A pure red powder, 1.06 g, was obtained.

Hardjito et al. (2002) favored the use of Luria Bertani (LB) agar medium (Difco) and overnight incubation at 30°C was used to maintain the strain. The medium employed to investigate temperature and pH relationship to pigment production contained beef extract (Lab-Lamco Powder, Oxoid) 3 g/L, peptone (Difco) 5 g/L and NaCl 5 g/L. Experiments were carried out using 250 mL flasks containing 100 mL medium adjusted to pH 7.0 and incubated with shaking (150 rpm), at 30°C. Growth temperatures used were 25, 30 and 37°C. The growth rate was defined as the slope of the regression line during exponential phase of growth.

LB media was again used but this time in the broth form for the revival and pre-culture of stock of *Serratia marcescens*. Binary combinations of the three components of LB broth were used for the batch culture of the strain to identify key components affecting cell growth and the production of the pigment. Also, LB broth was modified by adjusting the concentration of each component individually to determine the optimum medium formulation of pigment production by the culture strain. LB medium was also supplemented with three types of vegetable oils (2-6%). Batch culture was carried out at 30°C with an agitation rate of 200 rpm. Now for isolation culture broth was mixed with four-fold dilution of methanol and vortexed. The mixture was centrifuged at 10,000 x g for 10 minutes and the organic red portion was filtered through a 0.2μm filter paper. The filtrate was concentrated using a rotary evaporator and subsequently extracted with 3.0M chloroform. The chloroform phase was collected and re-concentrated to obtain a crude product. For purification, was dissolved in 20 ml of methanol and the solution was passed through a hexane-balanced silica gel column to trap the target product within the column. The loaded column was eluted with 10 M ethyl acetate to liberate the adsorbed product. The orange eluate was harvested and dried in a vacuum drier at 45°C to obtain the purified product in the form of a red powder (Wei and Chen, 2005).
According to Sundaramoorthy et al. (2009), a loopful of the *Serratia marcescens* culture was inoculated in pre-sterilized 100ml nutrient broth. The flask was kept in a shaker at 120 rpm for 16-18 hours at 30°C. The culture broth was centrifuged at 10,000 rpm for 20 min. Cell suspension was prepared using sterile distilled water and adjusted to 0.2 OD using UV Visible spectrophotometer. One percent (10^5 CFU/ml) of the above suspension was used as inoculum for the production of Prodigiosin. The bacterial isolate was sub-cultured in 100 ml of nutrient broth and incubated in a rotary shaker for 48 hours at 37°C. The organisms were harvested by centrifuging at 10,000 rpm for 10 minutes. The supernatant was discarded and the pellet was re-suspended in acidified ethanol (4% of 1M HCl in 96 ml ethanol). The mixture was vortexed and the suspension was centrifuged at 10,000 rpm for 10 minutes.

Column chromatography for separation of prodigiosin was also carried out and various fractions of prodigiosin were identified. A total of six fractions were reported readily separable by column chromatography (If), then an orange-red fraction (Ia), which subsequent re-chromatography showed to be comprised of two readily separable fractions; a pink fraction (Id and Ie) remained at the top of the column. After moving 5 to 10 cm down the column, the purple fraction (If) became stationary while the orange-red fraction (Ia) moved steadily down the column (Lynch et al., 1968). Someya et al. (2004) tested the amount of prodigiosin by scrapping 1 g of bacterial cells from LB agar plates and suspending it in 9 ml of ethanol. Prodigiosin was then extracted from the cells by shaking this suspension for 1 h followed by centrifugation. The supernatant was then filtered through a 0.20 µm filter (Toyo Roshi, Tokyo, Japan) and concentrated under reduced pressure at room temperature in the dark. The ethanol extracts were then further extracted using chloroform. The chloroform extracts were fractionated using LK5 silica gel thin-layer chromatography (Whatman International, Maidstone, UK), and the pigment spots were scraped from the plates and extracted with ethanol. The red pigment having an Rf value in the range of 0.90 to 0.95 was defined as prodigiosin.

2.11.5. Estimation of Prodigiosin:

One of the earlier prodigiosin estimation protocols was established by Haddix *et al.* (2000) according to which optical density values at 620 nm (OD620) could be
used to measure cell density without interference due to prodigiosin absorbance. *Escherichia coli*, another non-pigmented bacterium, produced an optical density profile similar to those for non-pigmented *S. marcescens* and *P. aeruginosa*. Further work identified the range of linear relationship between absorbance at 499 nm (A499) and pigmented cell concentration. This range was found to be approximately 0.0250 to 1.6000 absorbance. Similarly, OD620 varied linearly with non-pigmented or pigmented cell concentration in the approximate range of 0.0570 to 0.9850. Accurate quantification of dense cultures therefore required dilution into the 0.0570-0.9850 OD620 linear range.

The above information has allowed them to define a unit of prodigiosin expression based solely upon absorbance data from broth culture samples. First, they observed that the quotient A499 / OD620 was constant for each growth phase of non-pigmented *S. marcescens* Mutant 5 cells grown with aeration in nutrient broth plus maltose. Early log phase cells had an average ratio of 1.3267 (n = 12; s = 0.03728), late log phase cells showed an average ratio of 1.3955 (n = 11; s = .01537), and stationary phase cells had an average ratio of 1.4274 (n = 18; s = 0.01948). These three averages were themselves averaged to calculate the A499 / OD620 quotient across all phases of non-pigmented cell growth: 1.3831 (n = 3; s = 0.05134). The quantity (1.3831X OD620) therefore represented the contribution of cells alone, excluding prodigiosin, to the measured absorbance value at 499 nm. If the optical density due to cells alone was subtracted from the total absorbance at 499 nm, the expression obtained represented the absorbance due to prodigiosin alone:

\[A_{499} - (1.3831X \text{OD}_{620}) \text{ (expression 1)}\]

Finally, the absorbance due to prodigiosin could be divided by the optical density at 620 nm to express prodigiosin units on a per-cell basis. The factor 1000 was included to avoid working with numbers smaller than one:

\[\frac{[A_{499} - (1.3831X \text{OD}_{620})]}{\text{OD}_{620}} \times 1000 \text{ (Final equation)}\]

According to Hardjito *et al.* (2002) cell-bound and extracellular pigment was measured spectrophotometrically at 540 nm, and the total pigment was calculated by adding the cell-bound and extracellular values. A 5 ml broth was centrifuged at
10,000 rpm for 5 min. The extracellular pigment was determined directly from cell-
free supernatant at pH 5 by adding hydrochloric acid, while the cell-bound pigment
was extracted by adding 5 ml ethanol-chloroform (2:18).

Wei and Chen (2005), proposed a modified method for the estimation of
prodigiosin. The culture broth was mixed with an equal volume of 2% (w/v) alum
placed in a vial. 4 ml of methanol was added to the vial and the mixture was
vigorously vortexed. The solution was then centrifuged at 1200 x g for 10 minutes. A
fixed amount (0.8 ml) of the supernatant was further mixed with 0.2 ml of 0.05 N
HCl/methanol mixture (4:1 (v/v)). The optical density of the resulting solution was
measured at 540 nm. The OD \(_{540}\) was converted to mass concentration via appropriate
calibration using the purified prodigiosin like protein (PLP) product as the standard.

For the estimation of prodigiosin, Sundaramoorthy et al. (2009) used the
Slater et al. (2003) protocol. The organisms were harvested by centrifuging at 10,000
rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in
acidified ethanol (4% of 1M HCl in 96 mL ethanol). The mixture was vortexed and
the suspension was centrifuged at 10,000 rpm for 10 minutes. The supernatant was
transferred to a fresh vial and observed under UV-visible spectrophotometer at 534
nm. The prodigiosin produced was quantified using known concentrations of
prodigiosin.

The Haddix et al. (2000) equation was modified slightly by Mekhael and
Yousif (2008) where the new expression was

\[
\frac{\{A_{499} - (1.3831 \times OD_{620})\} \times 1000}{OD_{620}}
\]

This was used again by Samrot et al. (2011) in their paper to estimate prodigiosin
based on altering the nutritional and physical parameters.

2.11.6. Applications of Prodigiosin:

Prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosene) is a bacterial
metabolite that has anticancer and antimetastatic properties. However, the molecular
mechanisms responsible for these abilities are not fully understood. Gene expression
profiling of the human breast cancer cell line MCF-7 treated with prodigiosin was
analyzed by cDNA array technology. The majority of the significantly modified genes were related to apoptosis, cell cycle, cellular adhesion, or transcription regulation. The dramatic increase of the nonsteroidal anti-inflammatory drug activated gene 1 (NAG-1) made this gene an interesting candidate regarding the possible mechanism by which prodigiosin induces cytotoxicity in MCF-7 cells. Soto-Cerrato et al. (2007) showed that prodigiosin triggers accumulation of the DNA damage response tumor-suppressor protein p53 but that NAG-1 induction was independent of p53 accumulation. Moreover, prodigiosin caused AKT dephosphorylation and glycogen synthase kinase-3B (GSK-3B) activation, which correlated with NAG-1 expression. Prodigiosin-induced apoptosis was recovered by inhibiting GSK-3B, which might be due, at least partly, to the blockade of the GSK-3B-dependent up-regulation of death receptors 4 and 5 expression. These findings suggest that prodigiosin mediated GSK-3B activation is a key event in regulating the molecular pathways that trigger the apoptosis induced by this anticancer agent.

Park et al. (2008) carried out their research on the therapeutic potential of prodigiosins which further stimulated research into their mechanism of action. Here a possible relationship between the cytotoxicity of the prodigiosins and their DNA-damaging capacity was demonstrated. In the presence of redox-active metal cations, preferably copper$^{2+}$ (Cu(II)), prodigiosins facilitated single- (ss) and double-strand (ds) DNA cleavage. These events were thought to have been derive from formation of the π -radical cation at the electron-rich pyrrolylpyrromethene chromophore through interaction with Cu(II) to yield Cu(I), which fostered reductive-activation of molecular oxygen (O2) to form the superoxide radical anion (O$_2^\cdot$) and hence hydrogen peroxide (H$_2$O$_2$). The interaction of H$_2$O$_2$ with a Cu-bound prodigiosin species was thought to initiate dsDNA cleavage. Structure-activity relationships demonstrated that replacement of the individual metal-coordinating pyrrole rings by other weaker Cu (II)-ligating arenes resulted in marked loss of nuclease activity and cytotoxicity. Hence their study showed photo-induced cytotoxicity of the natural product prodigiosin 1 along with three structure analogues 2-4. The natural prodigiosin 1 was too cytotoxic in the dark to be considered an effective phototherapeutic agent even though it showed ∼3-fold enhancement in cytotoxic potency upon irradiation with visible light. Of the structural analogues tested, the derivative 4 showed similar potency to 1 in the presence of visible light, but unlike 1,
it failed to inhibit colony formation of leukemia (HL-60) cells in the dark. These results demonstrated the potential of prodigiosin- based compounds, such as 4, for cancer treatment by photodynamic therapy.

The antibacterial activity of prodigiosin was depicted in the work carried out by Mekhail and Yousif (2008). Their results indicated that the prodigiosin antibacterial activity was higher against gram positive bacteria including *Staphylococcus aureus, Staphylococcus saprophyticus, Bacillus subtilus, Enterococcus avium* and *Streptococcus pyogenes* as compared with gram negative bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Aeromonas hydrophila, Proteus mirabilis* and *Klebsiella pneumoniae*. The antibacterial activity of prodigiosin (PG) was the result of their ability to pass through the outer membrane and to their capacity for inhibiting target enzymes, such as DNA gyrase and topoisomerase IV, which inhibited the cell growth (Berlanga and Vinas, 2000).

Samrot et al. (2011) enhanced prodigiosin production to its optimum level and used the extracted pigment in the screening of antibacterial activity. Among all the extraction procedure, ethyl alcohol and HCl method was found to extract higher quantity out of *Serratia marcescens* as it was reflected on bioactivity. The zone of inhibition was measured as 16 mm against *E.coli*, 19 mm for *Pseudomonas sp.* and 20mm for *S. aureus*. The method used for observation of antibacterial activity was agar-well diffusion assay.

### 2.12.1. Protease

Proteases are enzymes that break the peptide bond that joins amino acids together in proteins. They are examples of hydrolases, enzymes that break a chemical bond by the addition of a water molecule. Although the hydrolysis of a peptide bond is energetically downhill, this process is very slow at normal temperature and pH (Neitzel, 2010). They are also known as proteolytic enzymes or proteinases that break the long chainlike molecules of proteins into shorter fragments (peptides) and eventually into their components, amino acids. Proteolytic enzymes are present in bacteria and plants but are most abundant in animals.
The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Rao et al., 1998).

2.12.2. Microbial Proteases

The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Godfrey and West, 1996). Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications.

Most commercial proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus Bacillus. Bacterial neutral proteases are active in a narrow pH range (pH 5 to 8) and have relatively low thermotolerance. Due to their intermediate rate of reaction, neutral proteases generate less bitterness in hydrolyzed food proteins than do the animal proteinases and hence are valuable for use in the food industry. Neutrase, a neutral protease, is insensitive to the natural plant proteinase inhibitors and is therefore useful in the brewing industry. The bacterial neutral proteases are characterized by their high affinity for hydrophobic amino acid pairs. Their low thermotolerance is advantageous for controlling their reactivity during the production of food hydrolysates with a low degree of hydrolysis. Some of the neutral proteases belong to the metalloprotease type and require divalent metal ions for their activity, while others are serine proteinases, which are not affected by chelating agents. Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10, and their broad substrate specificity. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry.

2.12.3. Mechanism of action of proteases

The mechanism of action of proteases has been a subject of great interest to researchers. Purification of proteases to homogeneity is a prerequisite for studying
their mechanism of action. Vast numbers of purification procedures for proteases, involving affinity chromatography, ion-exchange chromatography, and gel filtration techniques, have been well documented. The catalytic site of proteases is flanked on one or both sides by specificity subsites, each able to accommodate the side chain of a single amino acid residue from the substrate. These sites are numbered from the catalytic site $S_1$ through $S_n$ toward the N terminus of the structure and $S_{19}$ through $S_{n9}$ toward the C terminus. The residues which they accommodate from the substrate are numbered $P_1$ through $P_n$ and $P_{19}$ through $P_{n9}$, respectively.

2.12.4. Protease from *Serratia marcescens*

*Lyerly and Kreger* (1979) reported that an extracellular, non-elastolytic, neutral metalloprotease of *Serratia marcescens* was obtained after extensive purification. This was further elucidated by *Ustáriz et al.* (2008) in their paper where the protease was described as potent extracellular metalloprotease, which is widely used as an anti-inflammatory agent. Alkaline protease production was determined under solid state fermentation by a certain strain of *Serratia marcescens* and maximum production was seen from wheat bran medium (*Joesph et al.*, 2011).

2.12.5. Mechanism of action of metalloprotease

The mechanism of action of metalloproteases is different from serine or aspartate proteases. These enzymes depend on the presence of bound divalent cations and can be inactivated by dialysis or by the addition of chelating agents. For thermolysin, based on the X-ray studies of the complex with a hydroxamic acid inhibitor, it has been proposed that Glu143 assists the nucleophilic attack of a water molecule on the carbonyl carbon of the scissile peptide bond, which is polarized by the $Zn^{2+}$ ion (*Holmes and Matthew, 1981*). Most of the metalloproteases are enzymes containing the His-Glu-Xaa-Xaa-His (HEXXH) motif, which has been shown by X-ray crystallography to form a part of the site for binding of the metal, usually zinc.

2.12.6. Screening and production of protease

Screening for protease is determined by growing the organism on skim milk agar incubated for 24 – 48 hours at 37°C. Solid state fermentation technique is the method used for the production of proteases. According to *Ustáriz et al.* (2008), the
cells used for inoculation were previously grown for 12 hours (logarithmic phase) in 250 ml Erlenmeyer flasks containing 100 ml of Nutrient Broth (NB) (Biokar Diagnostic) (20 g/L) enriched with glucose (10 g/litre) at 30°C and 200 rpm. Cells were separated from the broth by centrifugation at 13,000 g for 15 minutes, washed with distilled water and re-suspended in NaCl (9 g/litre). For the culture medium preparation, fresh sweet whey, supplied by an industrial dairy (Reny-Picot, Asturias–Spain), was employed as the culture medium. The initial protein concentration was varied between 6.0 g/litre and 12.0 g/litre and pH 6.1. In all cases, whey was sterilized by tangential microfiltration (0.33 µm at 1.5 bar), showing a decrease in the initial protein contents resulting in protein concentrations between 3.5 g/litre and 7.4 g/litre.

Two kinds of fermenters were employed: 250 ml capacity Erlenmeyer flasks containing 100 ml of fresh whey and a 5.0 litre capacity mechanically stirred bioreactor (Bioflo III, New Brunswick Scientific Co. Inc.), containing 2.6 litre of fresh whey. Erlenmeyer flasks were employed to study the effect of pH, temperature and initial protein concentration and the bioreactor to study the effect of dissolved oxygen concentration.

The production of proteases was carried out by solid state fermentation (SSF) method. Ground nut shell medium (sieved ground nut shell – 100 gms, Sodium chloride – 0.5 gms, Casein – 0.5 g, Distilled water -250 ml, wheat bran medium (sieved Wheat bran – 100 g; sodium chloride, casein – 0.5 g; Distilled water – 250 ml) and chemical medium (Glucose – 100 mg; peptone – 0.5 g; KH₂PO₄ - 0.5 g; MgSO₄.7H₂O – 0.2 g; FeSO₄.7H₂O) – 0.01 g; Casein digest – 1.5 g; Lactose – 2.0 g; Distilled water – 100 g) were used in this study. The contents of the flask were inoculated with 1 ml of inoculums (1x10⁸ cells/mL) after autoclaving. The contents were mixed thoroughly by gently beating the flasks on the palm of the hand and incubated in different incubation temperatures (12, 24, 36, 48 and 72 hrs). The protocol adopted for the optimization of culture conditions influencing alkaline protease production was to evaluate the effect of individual parameters. The parameters optimized were: (a) various incubation temperatures range from 25, 35, 40, 45, 50°C (b) Various pH conditions range from 6, 6.5, 7, 7.5, 8, 8.5 (Joseph et al., 2011).
2.12.7. Purification of protease

The following method is in accordance with the protocol designed by Lyerly and Kreger (1979). The *S. marcescens* culture was cultivated in tryptone-yeast extract-glucose broth (pH 7.2) containing 0.5% tryptone (Difco), 0.25% yeast extract (BBL), and 0.1% glucose. Aliquots (0.1 ml) of a stationary phase culture of the bacterium were added to 24 (2-litre) flasks containing 200 ml of broth, and the broth was incubated for 20 to 24 hours at 30°C on a gyratory shaker (model G-25, New Brunswick Scientific Co., New Brunswick, N.J.) operating at 200 to 210 cycles/ min. The culture supernatant fluids were obtained by centrifugation.

Ammonium sulfate was added slowly to the pooled culture supernatant fluids, with gentle stirring, to a final concentration of 60% saturation (420 g/liter). After 18 to 24 hours, the precipitate was recovered by centrifugation, washed once with 60% saturated ammonium sulfate (100 ml), and dissolved in 5 ml of 0.02 M potassium phosphate buffer (pH 7.0). The preparation was dialyzed overnight against 8 litres of phosphate buffer, and a small amount of insoluble residue was removed by centrifugation.

The above preparation was applied to a column (2.6 by 31 cm) of hydroxyapatite (Bio-Gel HTP, Bio-Rad Laboratories, New York, N.Y.) equilibrated with 0.02 M potassium phosphate buffer (pH 7.0). The column was washed, at a flow rate of 20 ml/h or 80 ml/h, with approximately 600 ml of equilibrating buffer, and fractions (10 ml) were collected. After washing the column, a single-step elution was performed, at a flow rate of 20 ml/h, with 0.3 M potassium phosphate buffer (pH 7.0), and fractions (5 ml) were collected. All fractions were assayed for absorbance at 280 nm and for protease activity, and fused rocket immunoelectrophoretic analysis of the fractions was performed. The protease peak fractions were pooled and concentrated to 15 ml by ultrafiltration in a stirred cell equipped with a PM 10 membrane (Amicon Corp., Lexington, Mass.), and the membrane was washed twice with 5 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.5). The pool of the concentrate and the membrane washings was dialyzed overnight against 6 liters of 0.01 M Tris-hydrochloride buffer (pH 7.5).

The stage 3 preparation was subjected to preparative electro-focusing in a flat bed of washed Sephadex G-75, superfine (Ultrodex; LKB Instruments, Inc..
A 4% (wt/vol) gel slurry containing the stage 3 preparation and the ampholine buffer mixture required to generate a linear pH gradient of 4.0 to 6.5 (2.5 ml each of pH 4 to 6 and 5 to 7 ampholines) was dried, at 10 to 12°C, to the required evaporation limit. Electrofocusing was performed for 18 to 19 h at a constant power of 7 W provided by the LKB 2103 power supply. The pH of odd-numbered gel fractions was determined with a surface glass electrode, and the supernatant fluids derived from mixing each gel fraction with 4 ml of 0.1 M AB were assayed for protease activity and analyzed by fused rocket immunoelectrophoresis. The protease peak fractions were pooled and concentrated to 5 ml by ultrafiltration with a PM 10 membrane, the membrane was washed twice with 2.5 ml of 0.1 M AB, and the concentrate and membrane washings were pooled.

The stage 4 preparation was applied to a column (2.6 by 96 cm) of Sephadex G-100 (regular) equilibrated with 0.1 M AB (pH 7.8), and was eluted, in the downward flow mode, at a flow rate of 20 ml/h. Fractions (5 ml) were assayed for absorbance at 280 nm and for protease activity, and were analyzed by fused rocket immunoelectrophoresis. Protease peak fractions were pooled and lyophilized, and the lyophilized preparation was stored at 0°C.

For obtaining cell-free extract, cells were harvested from the fermenter and centrifuged at 12 000 g for 10 min. The pellet was resuspended in 0.1 M Tris-HCl buffer, pH 7.2, and disrupted by sonication 3 times for 2 min at a frequency of 20 Kcycles s⁻¹, spaced by 30 s (MSE, 150 Watt ultrasonic desintegrator, England). After sonication, centrifugation was used to separate the particulate material and whole cells from a clear supernatant fluid, the intracellular medium (Henriette et al., 1993).

2.12.8. Estimation of protease

The final purified protease preparation (1 mg) was examined for the presence of hexoses, pentoses, and phosphorus, with glucose, ribose, and beta-glycerophosphate, respectively, as standards (Lyerly and Kreger, 1979). The absorbance, at 280 nm and 260 nm, of a 1-mg/ml solution (in 0.1 M ammonium bicarbonate [AB]) of the purified protease was measured with 10-mm light path cuvettes in a Beckman DB-GT spectrophotometer. Protein was estimated by the method of Lowry et al. (1951), with crystalline bovine serum albumin as the standard.
Cell-free supernatant fluids were obtained by centrifugation of 5 ml aliquots at 12000 g for 10 min at 4°C casein was dissolved at a concentration of 20 g/l, in a 0.1 M sodium phosphate buffer (pH 7.8) containing CaCl₂ at a concentration of 5 mM. 2 ml of this substrate solution were mixed with 1 ml of cell-free supernatant fluid and incubated at 40°C for 30 min. 6 ml of a 5 g/l trichloroacetic acid solution (0.3 N) were added and the tubes were left to stand for 30 minutes. The precipitated protein was separated by centrifugation at 5000 g for 15 minutes. The quantity of soluble peptides in the supernatant was estimated according to Lowry et al. (1951) at a wavelength of 500 nm. Units of enzyme activity (U) were calculated from a standard curve and expressed as µg of tyrosine released from casein per hour at 37°C. Specific activity was expressed as units of protease activity per mg of cell dry mass (Henriette et al., 1993).

Culture samples were centrifuged at 16,000 g for 15 minutes and the supernatant was retained for analysis. Total and specific protease activities on cell-free supernatant were spectrophometrically analysed by the Azocasein method. The reaction was carried out with 480 µl of Azocasein solution (10 g/L in tris-glycin buffer, 0.2 M, pH 9.0 and 2mM CaCl₂) and 120 µl of sample, at 45°C for 1 h. The reaction was stopped by adding 600 µl of trichloroacetic acid (100 g/L), the reaction mixture was centrifuged at 16,000 g for 15 min. A volume of 200 µl of NaOH (1.8 M) was then added over 800 µl of the supernatant and absorbance was measured at 420 nm. Class-specific inhibitors of serineprotease (phenylmethylsulfonyl fluoride (PMSF) 4mM) and metalloprotease (1, 10- phenanthroline 5mM) were employed to determine the specific kinds of proteases produced. The determination of the specific activities was performed by the addition of specific inhibitor to the sample and they were maintained at room temperature for 15 minutes before being assayed. All the samples with absorbance higher than 0.5 were diluted with tris-HCl buffer, 50 mM, pH 7.6 and analyzed once again. One unit of protease activity was defined as the amount of enzyme that causes an increase in absorbance at 420 nm of 0.1 units in one hour. Standard deviation of the data ranged between 0.2 EU/mL and 484 EU/mL. Total protein concentration of the cell-free culture supernatant was analysed by the Lowry Method. Bovine serum albumin was used as the standard (Ustáriz et al., 2008).
According to Joseph et al. (2011), after the fermentation, culture mass of solid medium was extracted with 1:10 volumes of distilled water (pH: 7) with shaking at 160 rpm for 1 h at 25-30°C, centrifuged and supernatant was used as the crude enzyme extract for the assay. Protease activity in culture supernatant was determined using spectrophotometric method. Enzymes solution (1.0ml) was incubated with 1.0ml of 2.0% (w/v) casein in phosphate buffer (50mM, pH 7.0) at 50°C for 10 min and then the reaction was terminated by the addition of 5.0 ml (5.0% w/v) aqueous solution of trichloroacetic acid. After 30 min of incubation at room temperature, the mixture was filtered and 2.0ml of filtrate was added to 4.0ml of 0.1N NaOH and 0.5ml of diluted folin-ciocalteau reagent. The tyrosine residues released by enzymatic hydrolysis of protein were determined. A separate blank was set up to correct the non-enzymatic release of tyrosine. One unit of enzyme activity was defined as the amount of enzyme required to release one µg of tyrosine per min with bovine serum albumin (BSA) as standard under the assay condition described. The values were calculated at 660 nm for enzyme and 760nm for protein.

2.12.9. Application of protease

Proteases have a large variety of applications, mainly in the detergent and food industries. In view of the recent trend of developing environmentally friendly technologies, proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes (Rao et al., 1998). The worldwide requirement for enzymes for individual applications varies considerably. Proteases are used extensively in the pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds, etc. Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations, whereas those that are used in medicine are produced in small amounts but require extensive purification before they can be used.

Detergent Industry: Proteases are one of the standard ingredients of all kinds of detergents ranging from those used for household laundering to reagents used for cleaning contact lenses or dentures. The use of proteases in laundry detergents accounts for approximately 25% of the total worldwide sales of enzymes. The key parameter for the best performance of a protease in a detergent is its pI. It is known that a protease is most suitable for this application if its pI coincides with the pH of
the detergent solution. Esperase and Savinase T (Novo Industry), produced by alkalophilic *Bacillus spp.*, are two commercial preparations with very high isoelectric points (pI 11); hence, they can withstand higher pH ranges. Due to the present energy crisis and the awareness for energy conservation, it is desirable to use proteases that are active at lower temperatures. All detergent proteases currently used in the market are serine proteases produced by *Bacillus* strains. Fungal alkaline proteases are advantageous due to the ease of downstream processing to prepare a microbe-free enzyme.

**Leather Industry:** The conventional methods of leather processing involve hazardous chemicals such as sodium sulfide, which create problems of pollution and effluent disposal. The use of enzymes as alternatives to chemicals has proved successful in improving leather quality and in reducing environmental pollution. Proteases are used for selective hydrolysis of non-collagenous constituents of the skin and for removal of non-fibrillar proteins such as albumins and globulins. The purpose of soaking is to swell the hide. Traditionally, this step was performed with alkali. Currently, microbial alkaline proteases are used to ensure faster absorption of water and to reduce the time required for soaking. The use of nonionic and, to some extent, anionic surfactants is compatible with the use of enzymes.

**Dairy Industry:** The major application of proteases in the dairy industry is in the manufacture of cheese. In cheesemaking, the primary function of proteases is to hydrolyze the specific peptide bond (the Phe105-Met106 bond) to generate para-k-casein and macropeptides. Chymosin is preferred due to its high specificity for casein, which is responsible for its excellent performance in cheesemaking. The proteases produced by GRAS (genetically regarded as safe)-cleared microbes such as *Mucor michei*, *Bacillus subtilis*, and *Endothia parasitica* are gradually replacing chymosin in cheesemaking. Bacterial proteases are also used to improve the extensibility and strength of the dough.

**Pharmaceutical Industry:** The wide diversity and specificity of proteases are used to great advantage in developing effective therapeutic agents. Clostridial collagenase or subtilisin is used in combination with broad-spectrum antibiotics in the treatment of burns and wounds. An asparginase isolated from *E. coli* is used to eliminate aspargine from the bloodstream in the various forms of lymphocytic leukemia. Alkaline
protease from *Conidiobolus coronatus* was found to be able to replace trypsin in animal cell cultures.

### 2.13.1. Lipase

Lipases, triacylglycerol hydrolases, are an important group of biotechnologically relevant enzymes and they find immense applications in food, dairy, detergent and pharmaceutical industries. Lipases are by and large produced from microbes and specifically bacterial lipases play a vital role in commercial ventures. Some important lipase-producing bacterial genera include *Bacillus*, *Pseudomonas* and *Burkholderia*. Lipases are generally produced on lipidic carbon, such as oils, fatty acids, glycerol or tweens in the presence of an organic nitrogen source. Bacterial lipases are mostly extracellular and are produced by submerged fermentation. The enzyme is most commonly purified by hydrophobic interaction chromatography, in addition to some modern approaches such as reverse micellar and aqueous two-phase systems. Most lipases can act in a wide range of pH and temperature, though alkaline bacterial lipases are more common. Lipases are serine hydrolases and have high stability in organic solvents (*Gupta et al.*, 2004).

### 2.13.2. Microbial Lipase

Although a number of lipase-producing bacterial sources are available, only a few are commercially exploited as wild or recombinant strains (*Palekar et al.*, 2000). Of these, the important ones are: *Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Burkholderia, Chromobacterium and Pseudomonas*. Of these, the lipases from *Pseudomonas* bacteria are widely used for a variety of biotechnological applications. Several products based on bacterial lipases have been launched successfully in the market in the past few years. A number of such products are from *Pseudomonas sp.*, such as Lumafast and Lipomax with their major application as detergent enzymes, while Chiro CLEC-PC, Chirazyme L-1 and Amano P, P-30 and PS have tremendous potential in organic synthesis.

### 2.13.3. Mechanism of action of lipase
A true lipase will split emulsified esters of glycerine and long-chain fatty acids such as triolein and tripalmitin. Lipases are serine hydrolases. Lipases display little activity in aqueous solutions containing soluble substrates (Sharma et al., 2001). Lipases are serine hydrolases which act at the lipid-water interface. The catalytic triad is composed of Ser-Asp/Glu-His and usually also a consensus sequence (Glyx-Ser-x-Gly) is found around the active site serine. The three-dimensional (3-D) structures of lipases reveal the characteristic α/β-hydrolase fold (Gupta et al., 2004).

2.13.4. Lipase from Serratia marcescens

Lipases from psychrotrophs, even at low concentration, can hydrolyze milk lipid and cause the rancid flavor in milk and dairy products that make these foods unacceptable to consumers. Lipase production by psychrotrophs varies with the species, as does the optimum temperature, optimum pH, and enzyme specificity (Abdou, 2003). Ray (1996) described Serratia species as gram-negative psychrotrophs that were able to grow and produce heat-stable extracellular lipase and protease during the refrigerated storage of foods. Some workers isolated psychrotrophs from refrigerated raw milk samples; about 3 to 6% of the isolated strains were S. marcescens (Abdou, 1997). Serratia marcescens was found to have lipolytic and proteolytic activities. Information on extracellular lipase produced by Serratia species is particularly limited, although some strains have been isolated from raw milk.

2.13.5. Screening and production of lipase

Serratia marcescens was streaked onto spirit blue agar medium and lipase reagent and the plates were incubated at 6, 17, and 30°C for up to 14 days. The plates were observed after 6 hours and every 12 hours for the clearing of the blue or deep blue color around each streak. Lipolytic activities of S. marcescens, at different degrees of temperature, were compared by measuring the width (millimeters) of areas of clearing or areas of deep blue (Abdou, 2003).

Microbial lipases are produced mostly by submerged culture, but solid-state fermentation methods can be used also. Immobilized cell culture has been used in a few cases. Many studies have been undertaken to define the optimal culture and nutritional requirements for lipase production by submerged culture. Lipase
production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature, and the dissolved oxygen concentration. Lipidic carbon sources seem to be generally essential for obtaining a high lipase yield; however, a few authors have produced good yields in the absence of fats and oils (Sharma et al., 2001).

*Serratia marcescens* was sub-cultured on nutrient agar slopes and then in nutrient broth at 30°C for 24 hours. Aliquots of 150 µl were inoculated into 15-ml portions of sterile 10% reconstituted skim milk and incubated at 30°C for 3 days, followed by incubation at 6°C for another 3 days. These portions were centrifuged at 20,000 × g at 4°C for 30 min. The supernatants were filtered using 0.45-µm cellulose acetate filter units (Toyo Roshi Kaisha, Ltd., Japan). The filtrates were used as the crude enzyme in the following experiments (Abdou, 2003).

After isolation of lipase producing bacteria from soil, water and other sources and its preliminary screening and biochemical identification, the pure culture of the lipolytic bacteria was maintained on nutrient agar medium. The extracellular lipase production was carried out in a medium composed of 3% w/v yeast extract, 1% w/v KH₂PO₄, 0.1% w/v MgSO₄.7H₂O, 0.5% w/v of maltose, 0.2% v/v of olive oil at pH 7.2. Medium was sterilized and inoculated with 1% seed inoculum prepared in nutrient broth followed by incubation at 37°C for 48 hours at 160 rpm in a shaker incubator. The cells were then centrifuged at 10,000 rpm for 15 mins and the supernatant was used as crude preparation of lipase for further studies (Gupta, 2011).

2.13.6. Purification of lipase

Many lipases have been extensively purified and characterized in terms of their activity and stability profiles relative to pH, temperature, and effects of metal ions and chelating agents. In many cases, lipases have been purified to homogeneity and crystallized. Purification methods used have generally depended on nonspecific techniques such as precipitation, hydrophobic interaction chromatography, gel filtration, and ion exchange chromatography. Affinity chromatography has been used in some cases to reduce the number of individual purification steps needed (Woolley and Peterson, 1994).
According to Abdou (2003), various steps were formulated to purify the lipase enzyme. The crude enzyme was first applied to a column (20 × 2.5 cm) of CM-cellulose (Sigma Chemical Co.), which was pre-equilibrated with 10 mM sodium phosphate buffer pH 7.5. The lipase was allowed to bind to the gel for 2 hours at 4°C and was eluted with a linear gradient of Triton X-100 (0 to 1%; 200 ml). The flow rate was 60 ml/h, and fractions of 5 ml were collected.

The lipase-rich fractions were pooled and applied to a column (15 × 1.5 cm) of DEAE-cellulose (Sigma Chemical Co.), which was pre-equilibrated with 10 mM sodium phosphate buffer at pH 7.5. The lipase was allowed to bind to the gel for 1 h at 4°C. The column was washed thoroughly with the same buffer to remove unabsorbed material, which included Triton X-100 and was eluted with a linear gradient of NaCl (0.05 to 1 M; 300 ml) in the same buffer at a flow rate of 40 ml/h. The eluate was collected in 5-ml fractions.

The concentrate was then applied to a column (100 × 2.5 cm) of Sephadex G-150 (Pharmacia Fine Chemicals, Uppsala, Sweden), which was pre-equilibrated with 10 mM sodium phosphate buffer pH 7.5 and eluted with the same buffer. The lipase-rich fractions were pooled, stored at 2°C, and used as the purified lipase.

According to Saxena et al. (2003), most of the microbial lipases are extracellular and the fermentation process is usually followed by the removal of cells from the culture broth, either by centrifugation or by filtration. The cell-free culture broth is then concentrated by ultrafiltration, ammonium sulphate precipitation or extraction with organic solvents. About 80% of the purification schemes attempted thus far have used a precipitation step, with 60% of these using ammonium sulphate and 35% using ethanol, acetone or an acid (usually hydrochloric) followed by a combination of several chromatographic methods such as gel filtration and affinity chromatography. Precipitation is usually used as a fairly crude separation step, often during the early stages of a purification procedure, and is followed by chromatographic separation. Increase in lipase activity depends on the concentration of ammonium sulfate solution used. Large quantities of material can be handled, and this step is less affected by interfering non-protein materials than chromatographic
methods. In comparison to other techniques, which give lower yields (60–70%), precipitation methods often have high average yield (87%).

Chromatographic steps: Ion exchange chromatography is the most common chromatographic method; used in 67% of the purification schemes analysed and in 29% of these procedures, it is used more than once. The most frequently employed ion-exchangers are the diethylaminoethyl (DEAE) group in anion exchange (58%) and the carboxymethyl (CM) in cation exchange (20%). Strong ion exchangers based on triethylaminoethyl groups and Q-Sepharose are becoming more popular in lipase purification. Gel filtration is the second most frequently employed purification method, used in 60% of the purification schemes and more than once in 22% of them. Affinity chromatography has been used as a purification step in 27% of the schemes. Hydrophobic interaction chromatography has been used in 18% of the cases with the most popular hydrophobic adsorbents being octyl or phenyl functional groups. The chromatographic methods chosen to purify microbial lipases depend on the initial lipase preparation, and even for the same crude lipase preparation, different purification schemes have been used. The choice of a purification method largely depends on its use in a particular stage of the total purification scheme. Some novel purification technologies have recently been applied to the purification of lipases. These include membrane processes, immunopurification, hydrophobic interaction chromatography employing epoxy-activated spacer arm as a ligand and polyethylene glycol–Sepharose gel, poly(vinyl alcohol) polymers as column chromatography stationary phases and aqueous two-phase systems.

2.13.7. Estimation of lipase

Activities of the crude lipase and chromatographic fractions were determined except that the substrate was tributyrin emulsion composed of 2% gum arabic, 0.4 M NaCl, and 5 mM CaCl₂ blended for 2 min at 50°C with the appropriate concentration of tributyrin in the suitable buffer (Abdou, 2003). A quantity (0.3 ml) of the enzyme preparation was added to 2 ml of the substrate solution in glass-stoppered test tubes that had been pre-incubated in a water bath at 37°C for 6 min at pH 8. The mixture was held for 10 min at 37°C. The enzymatic reaction was terminated by adding 7.5 ml of the extraction reagent (isopropane, 100 ml; heptane, 100 ml; 1 N H₂SO₄, 8 ml). After vigorous shaking for 2 min and standing for 1 hour, the upper layer obtained
was mixed with the color reagent (water 1 ml, phenol red 5 mg, Na-barbital 25 mg, ethanol 200 ml), and shaken for 30 seconds. The absorbance was measured at the wavelength of 420 nm (double-beam spectrophotometer UV-190°, Shimadzu Corporation, Kyoto, Japan). Optimum assay conditions (activity calculated as 100% activity) were 2 ml of 2% tributyrin emulsion mixed with 0.3 ml of lipase preparation maintained at 37°C in buffer of pH 8 for 6 min. One lipase unit activity was defined as the µ moles of liberated free fatty acid/min at 37°C. Palmitic acid was used as a standard fatty acid.

Gupta et al. (2011) stated in their research article that they followed the titrimetric method for determining lipase activity in the culture supernatant according to Sadasivam and Manickam (1996). Lipase activity was measured by titration of the fatty acid released with 0.1 M NaOH using 0.1% alcoholic phenolphthalein as indicator. 1 unit of lipase activity was defined as the amount of enzyme releasing 1 mole of free fatty acid in 1 minute under standard assay conditions.

2.13.8. Application of lipase

Lipases are widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics, and pharmaceuticals. Lipase can be used to accelerate the degradation of fatty waste and polyurethane.

Detergent Industry: Because of their ability to hydrolyzes fats, lipases find a major use as additives in industrial laundry and household detergents. Detergent lipases are especially selected to meet the following requirements: (1) a low substrate specificity, i.e., an ability to hydrolyze fats of various compositions; (2) ability to withstand relatively harsh washing conditions (pH 10–11, 30–60 ⁰C); (3) ability to withstand damaging surfactants and enzymes [e.g., linear alkyl benzene sulfonates (LAS) and proteases], which are important ingredients of many detergent formulations. In 1994, Novo Nordisk introduced the first commercial recombinant lipase ‘Lipolase,’ which originated from the fungus Thermomyces lanuginosus and was expressed in Aspergillus oryzae (Sharma et al., 2001).
**Food Industry:** The nutritional and sensory value and the physical properties of a triglyceride are greatly influenced by factors such as the position of the fatty acid in the glycerol backbone, the chain length of the fatty acid, and its degree of unsaturation. Lipases allow us to modify the properties of lipids by altering the location of fatty acid chains in the glyceride and replacing one or more of the fatty acids with new ones. Lipase-based technology involving mixed hydrolysis and synthesis reactions is used commercially to upgrade some of the less desirable fats to cocoa butter substitutes. Microbial lipases are used to obtain PUFAs from animal and plant lipids such as menhaden oil, tuna oil, and borage oil. Free PUFAs and their mono- and diglycerides are subsequently used to produce a variety of pharmaceuticals including anticholesterolemics, antiinflammatories and thrombolytics.

**Pulp and Paper Industry:** Lipases are used to remove the pitch from the pulp produced for paper making. Nippon Paper Industries, Japan, have developed a pitch control method that uses the *Candida rugosa* fungal lipase to hydrolyze up to 90% of the wood triglycerides.

**Ester Synthesis:** Lipases have been successfully used as catalysts for synthesis of esters. The esters produced from short-chain fatty acids have applications as flavoring agents in the food industry. Methyl and ethyl esters of long-chain acids have been used to enrich diesel fuels. *From et al.* (1997) studied the esterification of lactic acid and alcohols using a lipase of *C. antarctica* in hexane. Esterification of five positional isomers of acetylenic fatty acids (different chain lengths) with n-butanol was studied by Lie et al. (1998), using eight different lipases. *Krishnakant and Madamwar* (2001) reported using lipase immobilized on silica and microemulsion-based organogels, for ester synthesis.

2.14.1. Chitinase

Chitin is an unbranched polysaccharide composed primarily of 3-1, 4 linked N-acetyloglucosamine and occasionally glucosamine residues. It makes part of outer arthropod skeletons and also is one of components of fungi and some yeast's cell walls (*Knorr, 1984*). Chitin enzymatic hydrolysis for its component monomers runs with the assistance of specific chitinolytic enzymes. Chitinolytic enzymes are produced by a large group of organisms: fungi, bacteria, vertebrates, invertebrates and plants (*Donderski and Trzebiatowska, 1999*).
Chitin, a 1, 4-β-linked polymer of N-acetyl-β-D-glucosamine (GlcNAc), is the second most abundant polymer in nature, after cellulose. It is a major structural component of the exoskeleton of insects and crustaceans and it occurs in the cell walls of a variety of fungi. In accordance with the abundance of chitin, chitin degrading enzymes are found in a variety of organisms, varying from prokaryotes to man. In chitin-containing organisms, chitinases play an important role in normal life cycle functions such as morphogenesis and cell division, whereas plants produce chitinases as part of their defence against fungal pathogens. Many bacteria and fungi contain chitinolytic enzymes to convert chitin into compounds that can serve as energy source (Brurberg, 2000).

2.14.2. Chitinase from *Serratia marcescens*

The Gram-negative bacterium *Serratia marcescens* secretes a variety of extracellular enzymes including chitinases. *S. marcescens* is one of the most effective bacteria for degradation of chitin. When this bacterium is cultivated in the presence of chitin, a variety of chitinolytic enzymes and chitin-binding proteins can be detected. The precise number of different enzymes is somewhat difficult to determine on the basis of biochemical studies only, because some of the enzymes occur in multiple forms, e.g. on an SDS-PAGE gel. The latter is because the chitinases have a multi-domain structure (Fig 3), which makes them sensitive for partial proteolytic degradation. Thorough studies by a number of groups clearly show that *S. marcescens* produces at least three chitinases (ChiA, ChiB, ChiC), a chitobiase and a putative chitin-binding protein (CBP21) (Gal *et al*., 1998). It is conceivable, but not certain, that these five proteins represent the complete chitinolytic machinery of the bacterium. The chitinolytic machinery of *S. marcescens* is of great interest because it is one of the best characterized chitinolytic machineries known to date. Recently determined crystal structures of ChiA, ChiB and the chitobiase provide detailed insight in how a natural set of chitinolytic enzymes may be built up.

2.14.3. Structure of chitinase enzyme

Biochemical analyses of proteins produced by chitin-induced *S. marcescens* have revealed a number of relevant compounds, identifiable by their apparent molecular mass on an SDS-PAGE gel (Brurberg, 2000). ChiA is produced as a 563-residue precursor, which is secreted from the cells with concomitant cleavage of an N-
terminal signal peptide. The resulting enzyme has 540 residues and a calculated molecular mass of 58.5 kDa. Apart from deletion of the N-terminal methionine residue, the primary product of the chiB gene does not seem to be processed, despite the fact that ChiB is exported in S. marcescens. Mature ChiB contains 498 residues and its calculated molecular mass is 55.4 kDa. The chiC gene gives rise to various variants of ChiC in S. marcescens and two variants in recombinant E. coli. Recombinant E. coli produces a full length variant (ChiC1) consisting of 479 residues and with a calculated molecular mass of 51.6 kDa. The other variant (ChiC2) is a C-terminally truncated variant of ChiC1 that consists of approximately 325 residues; these two reports present different conclusions concerning the precise length of ChiC2). In S. marcescens, ChiC is exported without concomitant cleavage of an N-terminal signal peptide. Instead, unspecific N-terminal processing of the protein seems to occur which results in production of several slightly different ChiC species lacking 8–12 of the N-terminal residues. Analyses of the primary sequences of ChiA, ChiB, and ChiC1, as well as comparisons of available three-dimensional structures show that these enzymes have a modular structure, something which is very common for enzymes degrading insoluble biopolymers such as chitin and cellulose.

All three Serratia chitinases contain a catalytic domain with the characteristics of a family 18 glycosyl hydrolase. In addition, each of the enzymes contains domains that are putatively involved in substrate binding. The sequence alignment of the catalytic domains is characterized by the occurrence of relatively large numbers of long insertions and deletions. Several of these relate to variation in the structure and position of the extra domains. A fourth enzyme involved in chitin degradation is the 95 kDa chitobiase, which belongs to family 20 of glycosyl hydrolases. This enzyme is produced as an 885-residue precursor that is exported with concomitant removal of a typical 27-residue N-terminal signal peptide. The chitobiase consists of four domains, several of which have unknown functions. The catalytic domain has the same overall fold as the family 18 chitinases, but the active site architecture is different.

Upon induction with chitin, S. marcescens produces at least one more protein which has no enzymatic activity but which binds to chitin. This chitin-binding protein with an apparent molecular mass of 21 kDa (CBP21) is produced as a 197 residue precursor, which is exported with concomitant cleavage of a typical 27-residue N-terminal leader peptide. The mature protein consists of 170 residues and has a
calculated mass of 18.8 kDa. The protein shares 45 % sequence identity with a chitin-binding protein from *Streptomyces olivaceoviridis* and its general characteristics (e.g. the presence of aromatic amino acids) resemble that of known cellulose-binding domains from cellulases.

### 2.14.4. Mechanism of action of chitinase

Chitinases breakdown chitin into a variety of products that include the deacylated oligomer chitosan (GlcNAc)$_n$, the disaccharide chitobiose (GlcNAc)$_2$, and the monomer N-acetylglucosamine *(Bansode and Bajekal, 2006)* which when produced as an extracellular enzyme by *Serratia*, is utilized for growth and nutrition.

### 2.14.5. Screening and production of chitinase

Seawater, sediment and marine animals were collected from the coastal area of Cheju Island, Korea. Samples were diluted 10 to 1,000 fold in sterile seawater and spread on chitin-containing minimal agar plates. After incubation for 2 weeks at room temperature, clear-hole forming bacteria were selected as the chitinase producer. Among the bacteria showing the chitinase activity, strain 98CJ11027 isolated from a bryozoa, was selected for the production and characterization of chitinase *(Park et al., 2000)*. The use if sea water was specifically to select marine chitinase producing bacteria, since these were halophiles.

The isolate 98CJ11027 was pre-cultured in the same medium as described above without agar for 3 days at 30°C with stirring at 140 rpm. The medium (500 ml) was inoculated with 1% of preculture in a 2-liter Erlenmeyer flask and incubated for 6 days at 30oC on a reciprocal shaker.

According to *Bansode and Bajekal (2006)*, isolation of chitin utilizers was done through enrichment in colloidal chitin agar medium. By visual analysis, well-separated colonies that showed a zone of clearance around them were transferred onto slants as pure cultures. Colonies showing large zones of clearance were selected as potent chitinase producers. For production of chitinase, colloidal chitin broths were inoculated with 1 ml of the spore suspension of the isolates and incubated in a rotary shaker incubator at 150 rpm at 37-40°C for 12 days. The culture was then centrifuged at 8000 x g for 20 minutes and the cell free suspension was saturated with ammonium sulphate (60-70%) and kept overnight at 4°C for enzyme extraction. The precipitates
were dissolved in phosphate buffer and then dialyzed through a dialysis membrane (molecular weight cut off at 12,000 Da) against distilled water at 4°C overnight. The stocks were then preserved at 0-4°C.

2.14.6. Purification of chitinase

Protein concentrations were measured according to Bradford’s method using bovine serum albumin as the standard. After cultivation, the cells were removed by centrifugation at 5,000 x g and 4°C for 20 min. According to Park et al. (2000), proteins in the cell-free culture broth (900 ml) were precipitated with ammonium sulfate (30%, w/v). The precipitate was obtained by centrifugation and suspended in 3 ml of 0.1 M citrate-phosphate buffer (pH 6.0). The suspension was eluted through a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column (1 by 6 cm) to remove low molecular compounds and then lyophilized. The enzyme sample was applied onto a Q-Sepharose (Pharmacia) column (1.6 by 13 cm) equilibrated with 30 mM Tris-HCl (pH 9.0). Proteins were eluted with a linear gradient of 0 to 1 M KCl in 30 mM Tris-HCl (pH 9.0) at a flow rate of 4 ml/min. The active fractions were pooled and lyophilized. The concentrated sample was dissolved in 200 ml of 25 mM Tris-HCl (pH 7.0) and loaded onto a Sephadex G-200 (Pharmacia) column (1.5 by 60 cm) equilibrated with 25 mM Tris-HCl (pH 7.0). Proteins were eluted with 25 mM Tris-HCl (pH 7.0) at a flow rate of 0.2 ml/min.

In 2001, a revised protocol was formulated specifically for a particular strain of *Serratia marcescens*. All the procedures were carried out at 4°C. After growth of *Ser. marcescens* strain in the chitin medium for 7 days, the culture medium was centrifuged at 10,000 g for 20 min to remove the cells and undegraded chitin. The culture medium (300 ml) was subjected to precipitation with ammonium sulphate to 75% saturation, stirred for 4 hours and dialysed. This protein concentrate was loaded 4 (approximately 11.0 mg) on a Sephadex G-100 (Sigma, St. Louis MO, USA) column (1.0 x 40.0 cm) previously equilibrated with 50 mmol l\(^{-1}\)citrate phosphate buffer, pH 6.2. Elution was done using the same buffer and fractions were collected at a flow rate of 4 ml h\(^{-1}\). Fractions (1.0 ml) were collected and assayed for chitinolytic activity using pNPGlcNAc, pNP-(GlcNAc)2 and pNP-(GlcNAc)3 as the substrates. Fractions showing maximum chitinolytic activity towards pNP-(GlcNAc)3 were individually concentrated using Amicon stirred cell concentrator, with an
ultrafiltration membrane having a molecular weight cut-off of 10,000. This concentrate was checked for protein purity by SDS-PAGE on a 12.0% polyacrylamide gel. The gel was silver-stained (Nawani and Kapadnis, 2001).

2.14.7. Estimation of chitinase

Chitinase activity was measured by determining the release of p-nitrophenol from p-nitrophenyl b-D-N-acetylglucosaminide (PNG) on the basis of the method of Roberts and Selitrennikoff (1988) with modification. 100 ml of enzyme solution was added to 100 ml of 10 mM PNG (Sigma) and 300 ml of 0.1 M citrate-phosphate buffer (pH 6.0). After incubation at 37°C, the reaction was stopped with the addition of 500 ml of 1 M Na2CO3. The amount of p-nitrophenol was measured at 405 nm. One unit of chitinase was defined as the amount of enzyme needed to release one mmol of p-nitrophenol per hour (Park et al. 2000).

According to Nawani and Kapadnis (2001), the chromogenic derivatives p-nitrophenyl-N-acetyl-β-D-glucosaminide (pNP-GlcNAc), p-nitrophenyl-β-DN,N'-diacetylchitobiose [pNP-(GlcNAc)2] and p-nitrophenyl-β-D-N,N',N'´-triacetylchitotriose [pNP-(GlcNAc)3] (Sigma) were used as substrates for determination of chitinolytic activity. A 50 µl volume of the enzyme was incubated with 50 µl 0.3% solution of respective substrate in 50 mmol l⁻¹ citrate phosphate buffer, pH 6.2, at 47°C for 30 minutes, followed by the addition of 2.5 ml 10 mmol l⁻¹ NaOH. The release of the chromophore p-nitrophenol from the substrates was determined by measuring the absorbance at 410. One unit of enzyme activity was defined as the amount that released one micromole of p-nitrophenol per minute under the specified conditions.

2.14.8. Application of chitinase

One of the first biotechnological applications of chitinases concerned their use in biocontrol of plant pathogens. S. marcescens cultures, its chitinases, and its chitinase genes have shown potential as biocontrol agents in a variety of experimental set-ups. A highly chitinolytic strain of S. marcescens was found to suppress the growth of Botrytis spp. in vitro. In a greenhouse setting, S. marcescens has been shown to control B. cinerea, Rhizoctonia solani, and Fusarium oxysporum f. sp. cyclaminis, all pathogens of cyclamen (Someya et al. 2000).
Similarly, *S. marcescens* controlled growth of *Sclerotinia minor*, the causal agent of basal drop disease, in lettuce grown in green house. The chiA and chiB genes from *S. marcescens* have been transformed into other bacterial species like *Pseudomonas fluorescens* and *E.coli* in an attempt to improve their ability to control fungal plant pathogens. When ChiA was combined with *Bacillus thuringiensis* or with low concentrations of the *B.thuringiensis* delta-endotoxin, a synergistic toxic effect was seen on insect larvae. Transgenic tobacco plants expressing high levels of *S. marcescens* ChiA exhibited increased tolerance to *R. solani* as compared to untransformed control plants (*Brurberg et al. 2000*).

2.15.1. Biosurfactant

Biosurfactants are amphiphilic 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 et al., 1999*). The reason for their popularity as high value microbial products is primarily because of their specific action, low toxicity, higher biodegradability, effectiveness at extremes of temperature, pH, salinity and widespread applicability, and their unique structures which provide new properties that classical surfactants may lack. Biosurfactants possess the characteristic property of reducing the surface and interfacial tension using the same mechanisms as chemical surfactants.

Unlike chemical surfactants, which are mostly derived from petroleum feedstock, these molecules can be produced by microbial fermentation processes using cheaper agro-based substrates and waste materials. During the past few years, biosurfactant production by various microorganisms has been studied extensively. Also various aspects of biosurfactants, such as their biomedical and therapeutic properties, natural roles, production on cheap alternative substrates and commercial potential have been recently reviewed (*Muthusamy et al., 2008*).

**Table 2.1**: Major biosurfactant classes and microorganisms involved

<table>
<thead>
<tr>
<th>Surfactant class</th>
<th>Microorganism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolipids</td>
<td></td>
</tr>
<tr>
<td>Biosurfactants</td>
<td>Microorganisms</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td>Rhamnolipids</td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>Trehalose lipids</td>
<td><em>Rhodococcus erithropolis, Arthobacter sp.</em></td>
</tr>
<tr>
<td>Sophorolipids</td>
<td><em>Candida bombicola, C. Apicola</em></td>
</tr>
<tr>
<td>Mannosylerythritol lipids</td>
<td><em>C. antartica</em></td>
</tr>
<tr>
<td>Lipopeptides</td>
<td></td>
</tr>
<tr>
<td>Surfactin/ituran/fengycin</td>
<td><em>Bacillus subtilis</em></td>
</tr>
<tr>
<td>Viscosin</td>
<td><em>P. fluorescens</em></td>
</tr>
<tr>
<td>Lichenysin</td>
<td><em>B. licheniformis</em></td>
</tr>
<tr>
<td><strong>Serrawettin</strong></td>
<td><strong>Serratia marcescens</strong></td>
</tr>
<tr>
<td>Phospholipids</td>
<td></td>
</tr>
<tr>
<td>Surface-active antibiotics</td>
<td></td>
</tr>
<tr>
<td>Gramicidin</td>
<td><em>Brevibacterium brevis</em></td>
</tr>
<tr>
<td>Polymixin</td>
<td><em>B. polymyx</em></td>
</tr>
<tr>
<td>Antibiotic TA</td>
<td><em>Myxococcus Xanthus</em></td>
</tr>
<tr>
<td>Fatty acids/neutral lipids</td>
<td></td>
</tr>
<tr>
<td>Corynomicolic acids</td>
<td><em>Corynebacterium insidibasseosum</em></td>
</tr>
<tr>
<td>Polymeric surfactants</td>
<td></td>
</tr>
<tr>
<td>Emulsan</td>
<td><em>Acinetobacter calcoaceticus</em></td>
</tr>
<tr>
<td>Alasan</td>
<td><em>A. radioresistens</em></td>
</tr>
<tr>
<td>Liposan</td>
<td><em>C. lipolytica</em></td>
</tr>
<tr>
<td>Lipomanan</td>
<td><em>C. tropicalis</em></td>
</tr>
<tr>
<td>Particulate biosurfactants</td>
<td></td>
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<tr>
<td>Vesicles</td>
<td><em>A. calcoaceticus</em></td>
</tr>
<tr>
<td>Whole microbial cells</td>
<td><em>Cyanobacteria</em></td>
</tr>
</tbody>
</table>

2.15.2. Classification of biosurfactants

Unlike chemically synthesized surfactants, which are usually classified according to the nature of their polar grouping, biosurfactants are generally categorized mainly by their chemical composition and microbial origin. Biosurfactants can be divided into low-molecular-mass molecules, which efficiently lower surface and interfacial tension, and high molecular-mass polymers, which are more effective as emulsion-stabilizing agents. The major classes of low-mass surfactants include glycolipids, lipopeptides and phospholipids, whereas high-mass surfactants include polymeric and particulate surfactants (Table 1). Most biosurfactants are either anionic or neutral and the hydrophobic moiety is based on long-chain fatty acids or fatty acid derivatives, whereas the hydrophilic portion can be
a carbohydrate, amino acid, phosphate or cyclic peptide (Rosenberg and Ron, 1999, Muthusamy et al., 2008).

2.15.3. Mechanism of action of biosurfactant

Bacteria degrade and use n-alkanes and polycyclic aromatic hydrocarbons (PAHs) as carbon substrates in the presence of synthetic substrates more efficiently than without the use of surfactants. Emulsification, a consequence of lower interfacial tension between the hydrophobic and hydrophilic phases of oil in water, produces small aggregations of the hydrophobic molecules. This increases the surface area available for microbial interaction which in turn increases the rates for uptake and utilization by microorganisms. Bacterial surfactants may be components of the outer surface of the cell or they may be freely expressed into the medium (Lepo et al., 1997).

2.15.4. Biosurfactant in Serratia marcescens

The type of biosurfactant produced by *Serratia marcescens* falls under the classification of a lipopeptide called *serrawettin* (Muthusamy et al., 2008). Serrawettins are non-ionic biosurfactants and is comprised of three molecular species, serrawettin W1 cyclo (D-3-hydroxydecanoyl-L-seryl); W2, D-3-hydroxydecanoyl-D-leucyl-L-seryl-L-threonyl-D-phenylalanyl-L-isoleucyl lactone; W3, cyclodepsipeptide composed of five amino acids and one dodecanoic acid, have been reported (Matsuyama et al., 2011).

2.15.5. Screening and production of biosurfactant

For marine biosurfactant producing bacteria, the samples were spread on nutrient agar (Difco, USA) dissolved in seawater and incubated at room temperature for 1-2 days. After incubation, plates were enumerated and morphologically different bacteria were selected for biosurfactant screening (approximately 15 to 30 isolates per plate) and purified by re-streaking twice. Isolated colonies were inoculated into 100 ml of Marine Broth 2216 (Difco, USA) containing 2 drops of weathered crude oil and incubated with continuous shaking (200 rpm) for 24-48 hours at room temperature using a shaker (GFL, Burgwedel, Germany). Colonies possessing biosurfactant producing activity, as evidenced by emulsification of weathered crude oil, were chosen. In addition, the cell suspensions of isolated strains, cultivated without
weathered crude oil, were then tested for the presence of surfactant by using haemolytic activity, the qualitative drop collapsing test, and quantitative oil displacement test and emulsification activity (Maneerat and Pheetrong, 2007).

According to Techaoei et al. (2007), microorganisms from the soil samples were isolated from liquid enrichment cultures containing 0.1% soy bean oil as a carbon source. One gram of soil sample was incubated into 100 ml of culture medium. The Mckeen medium (20 g l⁻¹ glucose, 5.0 g l⁻¹ glutamic acid, 1.0 g l⁻¹ K₂HPO₄, 1.02 g l⁻¹ MgSO₄, 0.5 g L⁻¹ KCl) supplemented with 1 ml of trace elements solution (0.5 g l⁻¹ MnSO₄·7H₂O, 0.16 g l⁻¹ CuSO₄·5H₂O and 0.015 g l⁻¹ FeSO₄·7H₂O) adjusting to pH 7.0 was used as cultural medium. The cultures were incubated on rotary shaker (150 rpm) for 3 days at 45 °C (for the hot spring soil samples) and 30°C (for garage sites and culture collection strains). The culture suspension was screened for biosurfactant production by oil spreading test.

For oil spreading test, the selected strains were compared by measuring of the diameter of the clear zones occurred when a drop of a biosurfactant-containing solution is placed on an oil-water surface. The 50 ml of distilled water was added to a large Petri dish (15 cm diameter) followed by the addition of 20 µl of crude oil to the surface of water, 10 µl of supernatant of culture broth. The diameters of clear zones of triplicate assays from the same sample were determined. The emulsifying capacity was evaluated by an emulsification index (E₂₄). The E₂₄ of culture samples was determined by adding 2 ml of kerosene and 2 ml of the cell-free broth in test tube, vortexed at high speed for 2 min and allowed to stand for 24h. The E24 index is given as percentage of the height of emulsified layer (cm) divided by the total height of the liquid column (cm). The percentage of emulsification index calculated by using the following equation:

\[ E_{24} = \frac{\text{Height of emulsion formed}}{\text{Total height of solution}} \times 100 \]

To perform the parafilm M test, The 25 µl of bacterial supernatants when mixed with 1% xylenecyanol were added to the hydrophobic surface of parafilm M. The shape of the drop on the surface was inspected after 1 minute. The diameters of droplets were
evaluated. The sodium lauryl sulfate and phosphate buffer (pH 7.0) were used as a positive and negative control, respectively.

Isolated strains were screened on blood agar plates containing 5% (v/v) human blood and incubated at room temperature for 24 h. Haemolytic activity was detected as the occurrence of a define clear zone around a colony (Carrillo et al., 1996). Relationship between hemolytic activity and biosurfactant production in microorganisms is such that those which are hemolytic, in all probability produce biosurfactants as well. Results from screening methods revealed that hemolytic assays is not a reliable method to check biosurfactant production because hemolytic assay relies on lysis of blood cells, which can be caused by compounds produced by microbes other than the biosurfactants. In that case hemolytic assay may include strains that do not produce biosurfactant (Thavasi et al., 2011).

According to Rismani et al. (2006), one loop of bacterial culture isolated from a 24 hours old culture was inoculated in a 50 ml broth of M1 broth (1.25 g of yeast extract, 0.75 g of peptone, 15 g of agar per litre) in a 100 ml flask incubated at 25°C for 24 hours on a rotary shaker at 100 rpm. Inoculum with OD₆₂₀ = 0.1 (10⁷ bacteria ml⁻¹) (10%) was transferred into Minimal Salt Agar medium 100ml in a 500 ml flask on a rotary shaker for 5 days at 100 rpm.

The following year, a modified protocol was made for biosurfactant production. The culture broth was centrifuged at 10,000 rpm for at 4°C for 10 minutes and the biosurfactant was precipitated using 2 volumes of ethanol after setting the pH to 2.0 with 1:1 HCl. The precipitate was centrifuged and dried at room temperature. The concentration was expressed as g% or g l⁻¹ (Bidlan et al., 2007).

2.15.6. Purification of biosurfactant

After cultivation, a crude extract of the biosurfactant was obtained by centrifugation (10,000 x g, 15 minutes at 4°C) to remove the cells. The supernatant was precipitated with equal volume of chilled acetone and kept standing overnight at 4°C. The precipitate was collected via centrifugation and used for further purification using thin layer chromatography (Rismani et al., 2006).
According to Maneerat and Pheetrong (2007), bacterial cells were removed by centrifugation (12,000 x g, 4°C, 30 minutes). Culture supernatant was acidified with 6 N HCl to obtain the pH of 2.0. The extraction was performed twice with an equal volume of ethyl acetate. Pooled solvent extracts were concentrated using an evaporator under reduced pressure.

2.15.7. Estimation of biosurfactant

Surface tension of the cell-free broth was measured by a Du-Novy tensiometer (CSC No 70535, USA) at different time intervals. For determining the critical micelle dilutions the cell-free broth was diluted 10-fold (CMD\(^1\)) and 100-fold (CMD\(^2\)) with distilled water. The surface tensions of these dilutions were then plotted against time (Pruthi and Cameotra, 1997). Protein content of the biosurfactant was measured according to Lowry et al. (1951). Emulsification activity was also measured against various biofuels and hydrocarbons to test the emulsification index of the biosurfactant.

The sandpack test was performed according to the procedure described by Banat et al. (1991). Glass columns (40.0 x 2.5 cm) were packed with acid-washed sand saturated with 100 ml of oil. The efficiency of the isolated biosurfactant in releasing the oil was tested by adding 100 ml of biosurfactant solution (0.1%) to the column. Recovery of the oil was estimated by measuring the volume of the oil released.

2.15.8. Application of biosurfactant

According to Pruthi and Cameotra (1997), their Serratia marcescens biosurfactant was effective in producing excellent emulsification with decane, pristane, kerosene oil, motor oil and crude oil. These hydrocarbons showed emulsification activity of 98%, 100%, 100%, 94% and 85% respectively. The emulsion formed was stabilized by the surfactant and did not revert to separate oil and water phases even after 90 days. This biosurfactant, therefore, had dramatic emulsification capacity in addition to its surfaceactive properties.

To study the possible commercial applications of the precipitated biosurfactant in microbially enhanced oil recovery (MEOR), a sandpack column was prepared with acid washed sand. The precipitated biosurfactant (0.1% aqueous solution) was
effective in recovery of oil from a sandpack column saturated with known amounts of different hydrocarbons. It was demonstrated that as a result of biosurfactant action 90%, 85%, 82%, 80% and 80% of kerosene oil, nparaffin, motor oil, tank bottom sludge and Assam crude oil could be efficiently recovered from the column. It was also observed that a similar biosurfactant solution could remove crude oil from the walls of containers.

Many microorganisms have been reported to produce biosurfactants which have application in oil industries for petroleum production as well as for the incorporation of oil formulations, oil spill bioremediation / dispersion both inland and at sea, removal / mobilization of oil sludge from storage tanks, enhanced oil recovery, for emulsion polymerization for paints, paper coatings and industrial coatings, in cement, textile and fibre manufacturing, metal treatment, mining, water treatment, coal slurry defoamers and as wood preservatives (Bidlan et al., 2007).

In metal contaminated soils, biosurfactants show a lot of promise in removal of these contaminants. The interactions between surfactants and metals are not fully understood. It is known that surfactants can remove metals from surfaces by different mechanisms. Non-ionic metals can form complexes with biosurfactants, enhancing their removal from porous media. Anionic surfactants interact with cationic metals leading to their de-sorption from surfaces. Nevertheless, also cationic surfactants can play a role by competitive binding to negative charged binding sites.

The prospects of using biosurfactants in hydrocarbon–contaminated soil washing depend on the capacity of these compounds to enhance the desorption and dissolution of the polluting organic compounds and increase the rate of transport of contaminants in soils. The mechanisms involved in the hydrocarbon removal from soils are related to the mechanisms involved in increasing bioavailability for bioremediation purposes. The properties of stabilizing oil/water emulsions and increasing hydrocarbon solubility may enhance both the biodegradation rate and the hydrocarbon removal rate from soils. These mobilization and solubilization effects occur at both concentration below and above the CMC (critical micelle concentration). The more commonly studied biosurfactants, such as rhamnolipids and surfactin, have been successfully evaluated in washing of soils contaminated by crude oils, PAHs and chlorinated hydrocarbons (Franzetti et al., 2008).