Expression and Purification of Recombinant Pneumococcal Surface Proteins from *E.coli*

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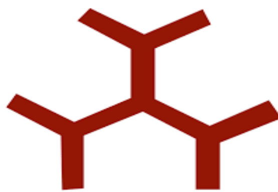

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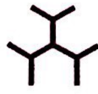

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DECLARATION

I solemnly declare that the work presented in my report entitled, **"Expression and Purification of Recombinant Pneumococcal Surface Proteins from *E.coli*"** is an authentic record of my work carried out under the supervision of Dr.A.K Panda, Director, National Institute of Immunology, New Delhi. I further declare that the contents presented in this report have not been submitted by me in any other university/ institute for the award of any degree, diploma, or other qualification.

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LIST OF ABBREVATIONS

Abbreviations	Word (s)
g	gram
ml	millilitre
L	Litre
w/v	Weight by volume
μg	Microgram
M	Molar
APS	Ammonium persulfate
TEMED	Tetramethylethylenediamine
IPTG	Isopropyl-β-D-thiogalactopyranoside
mM	Millimolar
rpm	Revolutions per minute
CD	Circular dichroism
PPV	Pneumococcal polysaccharide vaccine
PCV	Pneumococcal conjugate vaccine
OD	Optical density
kDa	kilodalton
nm	nanometre
PMSF	Phenyl methyl sulfonyl fluoride
BCA	Bicinchoninic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

ABSTRACT

Streptococcus pneumoniae is a gram-positive bacterium that causes invasive disease like pneumonia in lungs, bacteraemia in blood and meningitis in the central nervous system. Youngest and oldest segments mostly get affected by the disease in both developed and developing nations. The worldwide presence of pneumococcal resistance to antibiotics like penicillin complicates the therapy of pneumococcal infection. There are two vaccines available to address the worldwide pneumococcal disease burden, the 13-valent conjugate vaccine and the 23-valent non-conjugated polysaccharide-based vaccine. However, these two vaccines have limitations due to their capsular serotype- specific composition. Therefore, they elicit only serotype-specific immunity. As pneumococci can express diversified pool of capsular polysaccharide serotype, research is now centred on discovering more conserved protein- based antigen that could provide better defence against pneumococci. Present study focuses on exploring potential pneumococcal protein antigens as vaccine candidates. Pneumococcal surface adhesin A and SP0845 were the focus of this study.

1. INTRODUCTION

Streptococcus pneumoniae is an opportunistic, gram-positive, catalase-negative, commensal bacteria which colonises in the respiratory tract of most people. It causes both non-invasive diseases like pneumococcal conjunctivitis, otitis media and invasive diseases such as meningitis, sepsis, pneumonia. Pneumococcus colonization can even occur on the day of birth. Pneumococcal infection can spread from inoculum of either carrier or infected person. Based on the capsular polysaccharide composition and serotype reactions, 90 serotypes have been discovered. Within the age of one year, mostly children in the industrialized area get colonized with *Streptococcus pneumoniae*. It is main cause of morbidity and death. Pneumococcal carriage is prevalent in children and its occurrence is very high in developing nations compared to developed nations. Youngest and oldest segments mostly get affected by this disease. Around 1.2 million people a year as well as approximately 80,000 children, succumb to pneumococcal disease (Saxena *et al*., 2015). The capsule that envelops pneumococci is its key virulence element as it protects the bacterial against phagocytosis.

13-valent conjugated vaccine and 23-valent non-conjugated polysaccharide are the two vaccines which reduce the worldwide load of pneumococcal disease. These vaccines evoke antibodies to 13 and 23 capsular serotypes, respectively (Whitney *et al*., 2003). However, these two vaccines met with two major disadvantages:

- The non-conjugated polysaccharide vaccine failed to protect the young children and they do not generate an immune response mediated by T-cells.
- Conjugated vaccines have restricted coverage as they are based upon limited no of polysaccharides. Unless heavily subsided, they are too expensive to be used in the parts of the world with greatest need.

Success of a vaccine depends upon many factors like efficacy, its ability to confer long lasting immunity with minimum number of doses, ease of delivery, stability of formulated vaccine and cost. As a result of advances in biotechnology, new generation vaccines are in progress which are based on purified protein subunits, recombinant proteins, or synthetic peptides.

Pneumococcal vaccines have drawbacks because of their capsular serotype specific composition. Both the polysaccharide vaccine (PPV) and conjugate vaccines (PCV) are serotype-based and coverage of all the serotypes is difficult using this strategy

(Pichichero *et al*., 2016). *Streptococcus pneumoniae* changes its serotype with time, so multiple serotype dependent vaccines may have limited applicability. Increasing number of serotypes is the major problem of the current vaccines for the complete treatment of disease. Replacement of serotypes in colonization and their ability to cause disease is the major concern for developing a vaccine against pneumococcal disease.

Initially, it was believed that pneumococcal capsule masks the other virulent factors and the antibody against antigens other than capsule polysaccharide are able to protect against pneumococcal infection. That is why different serotypes were considered as independent pathogens for immunity in human. As recent findings have challenged the primitive view as noncapsular virulent factors has their role at the different stage of the pathogen and even few has been seen on the surface of capsular bacteria. The initial stage of pneumococcal infection is nasopharyngeal colonization (Austrian., 1986). During colonization, various noncapsular pneumococcal components are involved in interaction with the host receptors.

The capsule that envelops *S.pneumoniae* and the highly conserved surface and sub surface proteins are its major virulence determinants. Pneumolysin, Pneumococcal surface adhesin A, Pneumococcal surface protein A, Iga1 protease all of them contributes to pneumococcal virulence (Rajam *et al*., 2008). Systematic survival of pneumonia is mediated by all these proteins along with its attachment and even internalization. Attachment and internalization into the nasopharyngeal epithelial cells are mediated by surface and subsurface proteins, whereas the protection against phagocytosis is provided by the capsule.

So as to generate more robust immune response and to cover all the possible serotypes of *S.pneumoniae*, these proteins can be used as an alternate to polysaccharide and conjugate vaccines. Moreover, they generate T-dependent immune response with immunological memory.

Present study focuses on exploring the potential pneumococcal protein antigens for generation of vaccines. Pneumococcal surface adhesin A (PsaA) and SP0845 were the focus of this study. The purification of these proteins was carried out using Ni-NTA chromatography. Characterization of proteins were done by circular dichroism and fluorescence spectroscopy.

Protective efficacy of these vaccine candidates along with different adjuvants can be evaluated in animal models. These protein antigen-based vaccines could serve as a serotype independent and cost-effective vaccine.

2. AIM AND OBJECTIVES

Streptococcus pneumoniae is a pathogenic bacterium that causes many diseases like pneumonia, bacteraemia and even meningitis. Pneumococcus infection is the key reason of morbidity and death among children and oldest segments of both developed and under developed countries. Present vaccines are capsular serotype based and therefore they elicit only serotype-specific immunity. PsaA and SP0845 are two highly conserved proteins of pneumonia with potential to offer broad protection. The aim of this study is-

- **Purification of pneumococcal surface proteins by affinity chromatography**
- **Characterization of purified protein by fluorescence and CD spectroscopy**

3. REVIEW OF LITERATURE

3.1 *Streptococcus pneumonia*

Streptococcus pneumoniae is gram-positive bacterial pathogen that occurs in pairs or short chains and is the major reason for causing serious illness including meningitis, pneumoniae, and bacteraemia in the young children and adults. *S. pneumoniae* is a commensal bacterium which colonizes in the nasopharyngeal niche. It was first isolated from the rabbit infected with the human carrier's saliva in 1881(Watson *et al*., 1993). Pneumococcal meningitis occurs both in adults and children while in children, pneumococci is the major reason for causing the otitis media. Kids below the 2 years, aged and immune compromised individuals mainly get infected with pneumococcus. Individuals with HIV, diabetes, asthma, pulmonary disease, sickle cell disease and cardiovascular disease are also at great risk for pneumococcal infection. About 30% of all adult pneumonia cases by pneumococcal infection are reported in developed countries and has morality rate of 11% to 40% (Bridy *et al*., 2005). Initial studies observed the rough and smooth morphology of pneumococci that were the characteristic of non-capsulated and capsulated strains, respectively (Avery *et al*., 2017). These findings guided the field of pneumococci and pneumococcal disease and provided the base for the research of capsulated strains only.

3.1.1 Serotype Identification

Pneumococcal strains were identified on the basis of variability in the molecular pattern of capsular polysaccharide. Pneumococcal classification was done by capsular typing for both non-capsulated and capsulated strains. Based on their genetic variations as well as antigenicity and biochemical characteristics, beyond 90 serotypes have been discovered (Bentley *et al*., 2006). Initially, a serological test (Quelling test) was performed for capsular typing which was invented by German bacteriologist Fred Neufeld in 1902. Nowadays, latex agglutination text is used most commonly (Slotved *et al*., 2004).

3.1.2 Pneumococcal colonization

Many bacterial species reside in the respiratory tract of the human beings. The nasopharyngeal flora of children is usually developed in the first few months of their existence (Faden *et al*., 1997). A diversity of microorganism counting *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis* can colonize the nasopharyngeal niche. Each person would probably be at least once in their lifetime

colonized with these pathogens. Pneumococcal carriage is prevalent in children and its occurrence is very high in developing nations compared to developed nations. Colonization rate is very high in the initial phase of life, which begins to decline from the age of 10. Poor mucosal immune response can result in constant and recurrent colonization and therefore infection, while powerful localized immune response to the pathogens will terminate colonization and deter delocalisation (Ghaffar *et al*., 1999). Additionally, hormonal changes during adulthood also lead to changes in nasopharyngeal microbiota and decrease in the rate of carriage with time. Although, these responses vary from one individual to another, the colonization rate is generally very low in adult person. Apart from the age group, there have been many risk factors such as high sugar level in the child's diet, children living in a group increase the acquisition of pneumococci. Studies reported that the tobacco smoke exposure also promotes to pneumococcal carriage and disease (Nuorti *et al*., 2000). Duration of colonization may vary from few days to several months. It is highly dependent on pneumococcal serotype present in the colony and the age of the host.

3.1.3 Mechanism of colonization

The polysaccharide capsule covers the outer surface of *S. pneumoniae.* Based upon capsular polysaccharides heterogenicity, more than 90 different capsular serotypes have been discovered. This capsule composed of polysaccharides is the key virulence element of *S. pneumoniae* as it guards the bacteria against phagocytosis (Watson *et al*., 1993). Reduced expression results in higher excess of the antibodies and complement to the pneumococcal surface, and thus enhancing clearance by the immune system. Capsular polysaccharides are highly immunogenic (Bogaert *et al*., 2004).

The cell wall, beneath the capsular layer is composed of polysaccharide and teichoic acid and acts as anchor for the cell wall mediated surface proteins. The intense inflammatory response is caused by the cell wall along with pneumococcal infection. This pneumococcal infection stimulates the influx of inflammatory cells which further initialize the complement system cascade and results in synthesis of cytokines (Bruyn *et al*., 1991). The capsule covering the cell wall protects cell wall from the host's response.

S. pneumoniae colonize the host's system by its attachment to the respiratory epithelial lining. Pneumococcus mainly colonize by linking to the N-acetylglucosamine (cell surface carbohydrates) on non-inflamed resting epithelium. Proteins like PsaA

expressed on the cell wall surface mediates the attachment of pneumococcus with these sugars (Moragues *et al*., 2003). The proteins expressed on the cell wall surface will also lead to the hydrophobic and electrostatic surface properties of pneumococcus and allow the attachment of host cells partly through non-specific, physio-chemical interactions (Swiatlo *et al*., 2002). Pneumococcus colonize through asymptomatic colonization and for it to convert from asymptomatic colonization to invasive disease, it requires generation of certain inflammatory factors like interleukin 1 and tumour necrosis factor (Tuomanen., 1997). This generation of inflammatory cascade changes the number and type of receptors on certain cells like epithelial and endothelial cells. For one of these upregulated proteins, the platelet activation factor receptor, pneumococcal cell wall choline demonstrates enhanced affinity. Binding to this receptor causes internalization of pneumococcus and induce transcellular migration through respiratory epithelium and vascular endothelium, which further results in invasion of live bacterium (Cundell *et al*., 1995). Variability in surface associated protein expression, structure or exposure could explain the colonization and invasion capability differences between strains.

With increased knowledge about mechanism of colonisation, surface-associated proteins are regarded prospective candidates for vaccines. While surface mediated-proteins like pneumolysin and PspA protect against systematic illnesses, PsaA and CbpA are promising applicants for colonization protection. According to the studies, to provide better defence against colonization and infection with *S. pneumonia,* a mixture of these proteins with different role in bacterial virulence can be used.

3.2 Pneumococcal Disease

Although pneumococci are usually colonized in the nasopharyngeal region, it can also other infectious diseases. It is responsible for otitis media in the inner ear as well as invasive diseases like pneumonia in lungs, meningitis in the CNS. Based on presence of pneumococcal infection in human body parts, pneumococcal diseases are broadly divided into the non-invasive and invasive diseases as shown in figure: 3.1. Non-invasive is usually located in specific area while the invasive disease is detected in sterile body fluids of a human. Pneumococci can be invasive when it enters the blood (Ludwig *et al*., 21012). Most incidences of pneumococcal diseases are found in developing nations such as Africa and South east Asia (Miyaji *et al*., 2103).

Pneumococcal disease was responsible for deaths in below 2 years children and the immune compromised people.

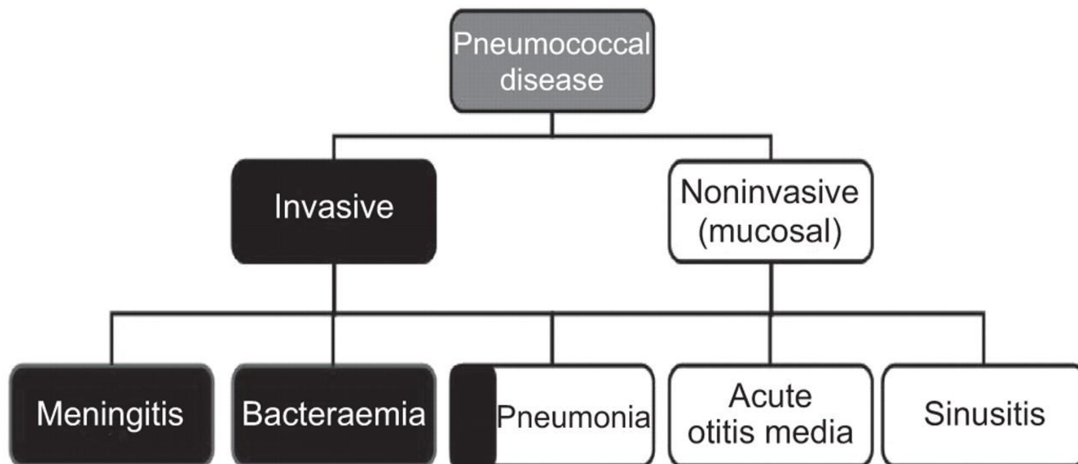


Figure 3.1: Types of diseases caused by pneumococci (adapted from Ludwig *et al*., 2012)

3.3 Prevention of pneumococcal infection

Two clinical inventions, antibiotics and vaccines are being used extensively to control the rates of nasopharyngeal carriage and infections caused by pneumococci. Despite the success of vaccines and antibiotics against pneumococcal infection, antibiotic resistance and emergence of non-vaccine serotypes have been the major cause of concern to combat pneumococcal disease. Discovery of the antibiotic penicillin by Alexander Fleming in 1928 began the antibiotic era. It showed broad-spectrum bactericidal activity and was very effective against the gram-positive bacterium such as Staphylococcus and Streptococcus. Soon after the success of penicillin, different classes of antibiotics including streptomycin, tetracycline, erythromycin, vancomycin, rifampicin, ciprofloxacin, and many more were developed for the treatment of various infectious diseases (Lewis., 2013). These antibiotics drastically reduced the number of deaths caused by many infectious diseases. But, with time antibiotic resistance was observed in many cases of infection.

The case of multidrug resistance in *S. pneumoniae* was first observed in 1977 and since than it has been growing rapidly (Whitney *et al*., 2000). Increasing numbers of antibiotic resistant pneumococci has been a global problem as available antibiotics may not be able to respond to infections.

3.3.1 Development of Vaccine

George Sternberg was one of the two scientists who first isolated the pneumococci independently in 1881 and showed that dead pneumococcus inoculated in rabbits were able to protect them from the bacterium injected later. This immunization in animals was the foundation for vaccine development. The first pneumococcal vaccine was developed from dead pneumococci of unknown identity in the early 1990s. It was administered in South African gold miner workers where pneumococci disease was very prevalent (Austrian., 1978). Although the cases of pneumococcus infection reduced after four months post vaccination, the vaccine was not protected over time. One of the possible hypotheses is the presence of non-vaccine serotype which caused infection in the vaccinated population. But it was very difficult to trace out the actual cause that time due to lack of knowledge about antigenic variations and their structures. Studies suggested that conjugate vaccines (composed of polysaccharide and proteins) confer better defence against *S. pneumoniae* than the vaccines which are composed of only one or limited mixtures of polysaccharides or protein components.

Commercially only two kind of pneumococcal vaccines are available to reduce the disease burden.

- Pneumococcal polysaccharide vaccine
- Pneumococcal conjugate vaccine

The encapsulated bacterial strain is harmful compared to the non-encapsulated ones. Therefore, the capsular covering is thought to be as the key virulence element of *Streptococcus pneumoniae*. It is the Capsule on the basis of which the bacteria are distinguished into more than 90 variant serotypes. Recently available 23-valent polysaccharide vaccine only elicits antibodies against capsular serotypes that are present in vaccine formulation.

Polysaccharide vaccine

The polysaccharide capsule was considered as the key virulence element of *Streptococcus pneumoniae*. Table 3.1 shows the serotypes present in the vaccine formulation, FDA approved dates of the vaccines and the effect of the vaccine on overall pneumococcal disease. Therefore, earlier the vaccine developed to provide protection against pneumococci contained the polysaccharide capsule as a vaccine candidate. The first vaccine developed against pneumococcus disease contained 14 capsular polysaccharide serotypes. In 1983, 23-valent polysaccharide vaccine, was the

first vaccine to be developed that provided protection against 90% of the pneumococcal capsular serotypes responsible for causing the disease. Currently available 23-valent polysaccharide vaccine contains mixture of following capsular serotypes: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F.

Table 3.1: Pneumococcal Vaccine Approval Data (adapted from Daniels *et al.*, 2016)

Vaccine	FDA approved	Serotypes present in vaccine formulation	Pneumococcal disease effect from vaccine serotypes
PPSV23	June 1983	1,2, *3,4,5,6B,7F,8, * 9V, 10A, 11A, 12F, 14, 15B, *17F, 18C, 19A, 19F, 20, * 22F, *23F and 33F*	1.Reduction invasive diseases 2. No effect on carriage
PCV7	February 2000	4, 6B, 9V, 14, 18C, 19F and 23F	1.Reduction invasive disease 2. Reduced carriage 3. Protective herd effect
PCV13	February 2010	1,3,4,5,6A, 6B,7F ,9V, 14,18C,19A, 19F and 23F	1.Reduction invasive disease 2. Reduced carriage

The vaccine is given either as a single dose or in multi-doses via intramuscular or subcutaneous mode of injection into the deltoid or lateral mild thigh. However, it shows some side effects like headache, fatigue, nausea etc. An individual above the age of 65 or older must receive a single dose of this (PPSV23) (Daniels *et al.*, 2016). Those individual at high risk or immune compromised must get single vaccination, before the age of 65. It is recommended that no person should get more than two booster doses PPSV23 before the age of 65, with a maximum duration of three booster doses. With PPSV23 vaccination, the invasive disease rate decreased in vaccinated individuals, however, the overall pneumococci carriage rate remained unaffected. The vaccine failed to generate immune response in kids below the age of 2 years. The polysaccharide vaccines generate T-cell independent immune response because of the reason that the repeating subunits of capsular polysaccharide can evoke an immune response in B-cells independent of T-cells. The vaccine has many advantages as well as disadvantages as

shown in table: 3.2. Therefore, to evoke more robust immune response and reduce the disadvantages of the polysaccharide vaccines polysaccharides conjugates vaccines were developed.

Table-3.2: Advantages and Disadvantages of 23-valent PS vaccine

Pros	Cons
1. The vaccine covers a large number of serotypes 2. Serotypes covered account for 85-90% of invasive pneumococcal disease 3. It is cost effective and widely used in developing countries	1.Cannot be used on children <2 years 2.Cannot be used on immunodeficient patients 3.Time period of protection is limited because of no T-cell response

Polysaccharide conjugate vaccine

With the aim to evoke immune response in kids before 2 years, polysaccharide conjugate vaccine was developed. The vaccine contained mixture of capsular polysaccharides along with diphtheria toxin. It was effective in generating T-cell dependent immunological response in kids below the age of 2 years. In 2002, the first conjugate vaccine i.e. 7-valent conjugate vaccine was developed that decreased the infection rates in kids below the age of 2 years (Hammitt *et al*., 2006). 13-valent polysaccharide conjugate vaccine was the second one developed to generate immune response against pneumococcus. This vaccine contained mixture of seven serotypes found in PCV7, five serotypes from PPSV23 and one unique serotype which is 6A. The enhanced coverage offered wider protection against infection with pneumococcal disease (Bryant *et al*.,2010). Later the implementation of 13-valent conjugate vaccine, invasive and non-invasive pneumococcal infection rates dropped.

Table-3.3: Advantages and Disadvantages of PVC-7 conjugate vaccine

Pros	Cons
1.More immunogenic than 23-valent PS vaccine 2.Can be used on infants under 2 years of age	1.Limited to the amount of polysaccharide-protein linkage that can be formed. 2.Not cost effective

3.4 Recent approach in pneumococcal vaccine development

Pneumococcal vaccine development efforts are now focused on proteins common to essentially all pneumococcal serotypes. This approach has several benefits, such as protein- based vaccine is expected to evoke immune response in all age groups, along with children below two years of age. Secondly, it is anticipated that these vaccines will be inexpensive and within the reach of developing nations. Thirdly, if extremely preserved proteins or protein epitopes are used as applicants for vaccine, wide and serotype autonomous protection can be anticipated. However, the function of protein present in vaccine formulation influence the type and degree of protection. The selection of well conserved proteins that play important role in bacterial survival and growth reduces the risk of negative selection caused by vaccination (Tai., 2006).

3.4.1 Pneumococcal Virulence antigens

Gram positive bacteria express certain proteins and enzymes on its surface as shown in the figure:3.2 which may contribute to pathogenesis and might be involved in the process of disease caused by these bacteria. However, these proteins may form direct linkages with the host tissue. Earlier, it was thought that the bacteria which do not have capsule covering are harmless whereas, those have capsular covering are the major virulence factor for *S. pneumoniae* (Olafsdottir *et al*., 2012). Current studies suggested that certain proteins like Pneumococcal surface adhesin A, Pneumococcal surface protein A, major autolysin, choline binding protein A, hyaluronate lyase could be considered as a capable vaccine.

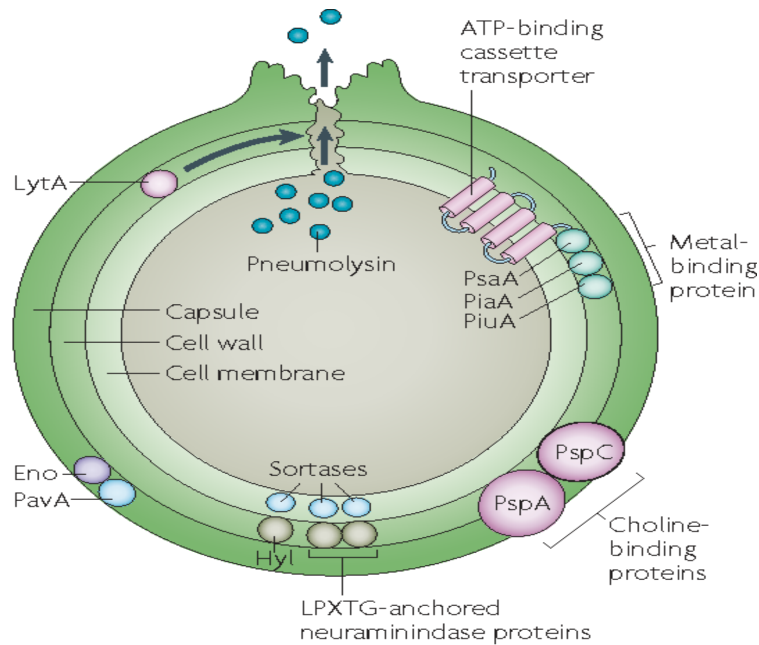


Figure 3.2: Pneumococcus virulence antigens (adapted from Kadioglu *et al*., 2008).

3.4.2 Pneumococcal protein antigens as potential vaccines

Pneumococcal surface adhesin A (PsaA)

PsaA is a pneumococcal lipoprotein expressed on its surface. It serves as a dual functional protein: transport of metal ions and adhesion of pneumococcal cells PsaA is a part of ABC transport system complex which transports Mn^{2+} ions into the pneumococcus. It is composed of 309 amino acids. PsaA is immediately lined to the cytoplasmic membrane and builds a covalent bond between the cysteine residue and diacylglycerol of the lipid bilayer (Rajam *et al.,* 2008).

PsaA is a surface-mediated, hydrophobic, immunogenic, and genetically conserved protein. Current studies revealed PsaA as an important component in pneumococcal vaccine formulation. Current studies are exploring PsaA to use it as an immunogen in the vaccine formulations.

SP0845

Streptococcus pneumoniae is the major reason for causing various diseases like pneumonia, sepsis, bacteraemia, meningitis and otitis media. As the currently available pneumococcal vaccines such as polysaccharides-based vaccines have met with certain limitations so to overcome them *S. pneumoniae* is being extensively studied for the production of protein-based vaccine. SP0845 is one the common protein antigens reported recently (Saxena *et al*., 2015). Size of this lipoprotein is 40kDa including a lipobox containing a signal peptide of 22 amino acids at N-terminus. It is a substrate

binding component of an ABC-transporter which is involved in nucleoside transportation and uptake of other substrates including essential nutrients like sugar and minerals (Saxena *et al*., 2015). SP0845 is highly conserved among pneumococcal serotypes and it shows very less identity to any human protein which is very important factor for considering it as a vaccine candidate.

4. MATERIALS AND METHOD

4.1 Requirements

- Bacto-Tryptone (Amresco, USA),
- Bacto-yeast extract (Difco, USA),
- Glycerol
- Sodium chloride (E. Merck India Ltd.)
- Ampicillin,
- Kanamycin,
- Isopropyl-β-D-thiogalactopyranoside (Sigma chemicals, USA),
- Sodium dodecyl sulfate (SDS) (Sigma Chemicals Co., USA)
- Tetramethylethylenediamine (TEMED) (Sigma Aldrich, Germany)
- Ammonium per sulfate (APS) (Sigma chemicals Co., USA).
- N,N-methylene bis-acrylamide (USB, Cleveland, UK)
- Phenyl methyl sulfonyl fluoride (PMSF) (Amresco)
- Methanol, glycerol and acetic acid (S.D fine-chem. Ltd., India).
- Bovine serum albumin (Sigma Chemicals India)
- Micro bicinchoninic acid protein assay reagent (Thermo scientific)
- Imidazole
- Mannitol

4.2 Equipments

- Electronic balance, BT 2245 (Sartorius Mechatronics, India).
- Homogenizer (PT 3100, POLYTRON).
- SDS-PAGE assembly (Amersham Pharmacia Biotech).
- Sonicator (BANDELIN)
- UV Visible Spectrophotometer (Uvikon@, Kontron Instruments,)
- Magnetic stirrer (Bellco, biotechnology, USA)
- Eppendorf Centrifuge 5810 (Eppendorf International, Germany)
- Vertical Laminar hood (Kartos International, Noida)
- Agarose beads for Ni-NTA column (QIAGEN, USA)
- Sorvall Evolution RC (Kendro Lab Products, Langenselbold, Germany)

4.3 Composition of Reagents used

(1) Luria Bertani (LB) media

- Tryptone 20 g
- Glucose 10 g
- Yeast Extract 10 g
- NaCl 20 g

(2) Lysis buffer (500ml)

- Tris 50 mM 12.5 mL
- NaCl 100 mM 2.9 g
- Mannitol 2% (w/v) 10 g
- PMSF 1mM 5.0 mL

(3) Electrophoresis buffer (1 liter)

Constituents	Quantity
Glycine	144.4 g
Tris base	30.3 g
Milli-Q	1000 mL
SDS	10.0 g

(4) Preparation of 12% Resolving gel

Name	10 mL	20 mL
Water	3.3	6.6
30% Acrylamide	4.0	8.00
1.5 M Tris pH 8.8	2.5	5.00
10% APS	00.1	00.2
10% SDS	00.1	00.2
TEMED	0.004	0.008

(5) Preparation of 5% stacking Gel

Name	**3 mL**	**6 mL**
Water	2.1	4.1
30% Acrylamide	0.5	1.00
1.5 M Tris pH 6.8	00.38	00.75
10% SDS	00.03	00.06
10% APS	00.03	00.06
TEMED	0.003	0.006

(6) Staining and destaining solution

Staining solution	Coomassie Brilliant Blue: - 0.2% in 5:4:1 of water, methanol and acetic acid respectively
Destaining solution	Water, methanol and acetic acid in 5:4:1.

METHODOLOGY

4.4 Expression and purification of recombinant Pneumococcal surface adhesin (PsaA).

A. Preparation of *E.coli* cells expressing target Protein

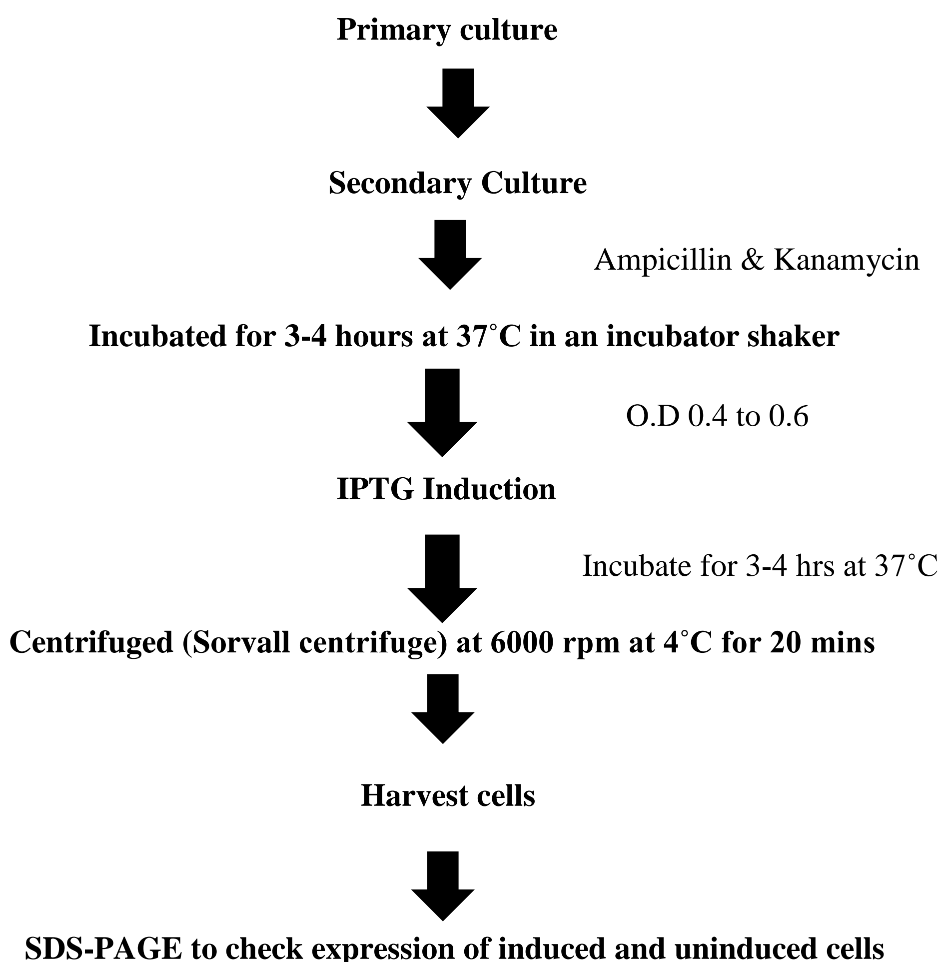
Preparation of Luria Bertani Media

All the media constituents were mixed and made the final volume to 2 L by addition of Milli-Q water. The media was autoclaved at 121°C at 15 psi.

Primary Culture of *E.coli* cells

20 mL primary culture was prepared under sterilized medium of Laminar air flow hood. 25 µg/mL of Kanamycin and 100 µg/mL of Ampicillin were added to it.

1mL glycerol stock of PsaA expressing *E.coli* cells was inoculated into the culture medium and incubated in an incubator shaker at 37˚C at 200 rpm for overnight.



Secondary culture of *E.coli* cells

To 500 mL of LB media, 5 mL primary culture was added along with kanamycin (250 µL) and ampicillin (500 µL). 1mL was aspirated separately as a blank. The secondary culture was incubated till 0.4 to 0.6 OD and then the culture was induced with 1mM IPTG. 1mL uninduced was aspirated separately before induction with IPTG. The induced secondary culture was further incubated in an incubator shaker for 3-4 hours at 37˚C.

Isolation of *E .coli* cells from culture media

E.coli cells have been pelleted down using from culture media with Sorvall centrifuge RC+ (Thermo Scientific, USA) at 6000 rpm at 4˚C for about 20 mins. The collected cells were analysed for expression of induced and uninduced cell through SDS-PAGE.

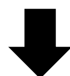
B. Growth Curve study

Two flaks were taken containing 1L LB media in each flask. Cells were allowed to grow until O.D reaches 0.4-0.6 at 600 nm. They were induced with 1mM IPTG and were grown for another 3 hours. Aliquots were taken every half hour. Then graph was plotted of OD against Time.

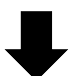
C. Preparation of cell lysates from *E.coli* cells

The pellet was suspended in 20mL lysis buffer and homogenized at 10,000 rpm for 2 minutes with a homogenizer. Cell homogenate was the sonicated for 2 cycles in ice and the resulting suspension was centrifuged at 11,500 rpm for about 30 mins. The supernatant was kept and the pellet was discarded.

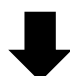
Cell pellet was suspended in 20 mL lysis buffer



Homogenized at 10,000 rpm for 2 min and sonicate 2 cycles



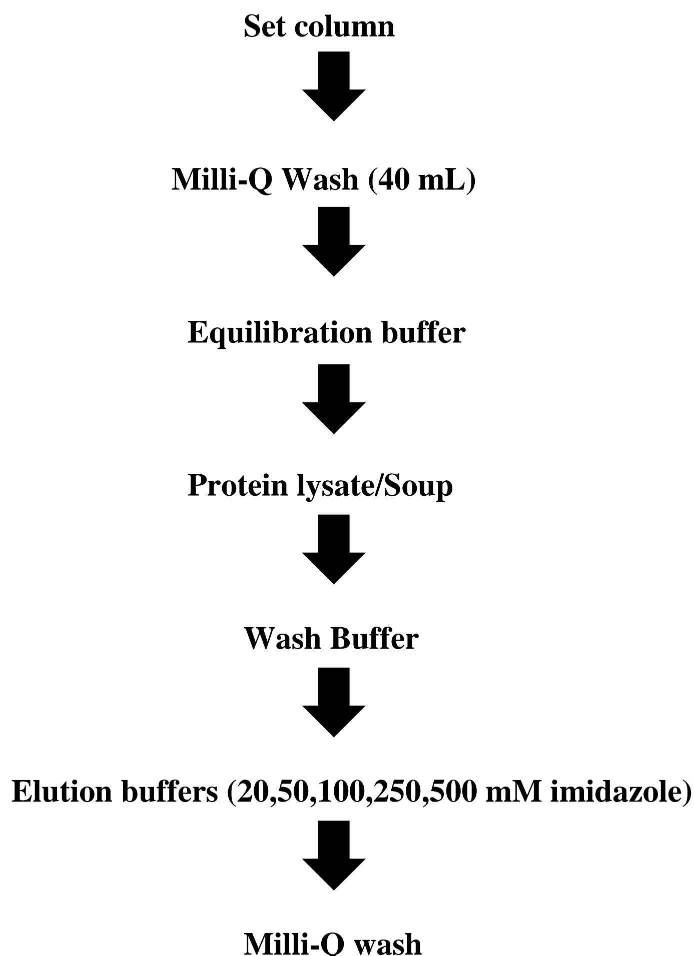
Centrifuge for 30 mins at 11,500 rpm



Keep supernatant at 4˚C

D. Isolation and Purification of his tagged protein by Ni-NTA Chromatography

The Ni-NTA chromatographic system is intended to purify 6x histidine tagged recombinant protein that are expressed in bacterial cells, insect cells, mammalian cells. The system is intended for recombinant proteins tagged with six tandem histidine residues around the elevated affinity and selectivity of Ni-NTA. Imidazole competes with histidine tagged for binding to the charged molecules and thus is used for elution of protein. Imidazole is usually added in increasing concentrations.



E. Gel electrophoresis for analysis of elutes collected by Ni-NTA chromatography

12% resolving gel and 5% stacking gel were prepared. Resolving gel was poured and polymerization was allowed. After that stacking gel was laid over the resolving gel and allowed to polymerize. The sample along with low molecular weight marker were loaded and allowed the gel to run at 80 mA for 2 hours.

Preparation of sample

Samples were taken in microfuge tubes and mixed loading dye and boiled for 2-3 minutes.

Staining the Gel

After running the samples, gel was transferred to staining dye for 1hour.

Destaining the Gel

After staining, the gel was shifted to the destaining dye for a minimum of 2 hours with at least three changes.

The elutes were separated and proceeded for desalting/buffer exchange.

F. Desalting/ buffer exchange

- Pure elutes after Ni-NTA affinity chromatography were pooled and dialyzed to remove the accessory salts (NaCl, Imidazole).
- A dialysis membrane (10 kDa cut off) was used for dialysis of protein.
- The dialysis process was performed in the 20 mM tris buffer, 2% mannitol.
- Dialysis was carried out in cold condition (4 °C).

G. Lyophilization of desalted proteins

The dialyzed protein was lyophilized using lyophilizer for 18 hours. After lyophilization the samples were collected and proceeded further.

H. Quantification of protein by BCA assay

- Lyophilized protein was dissolved in 1% SDS solution. Then samples were diluted for the analysis of total protein concentration using bicinchoninic acid assay.
- Standard curve was made in the range of 20 µg-200 µg/mL of BSA solution and concentration of the unknown sample was estimated.

I. Characterization of purified Protein

Characterization by Fluorescence spectroscopy

The Fluorescence spectra of the purified protein was carried out using Cary Eclipse spectrofluorometer. Fluorescence Spectrophotometer was calibrated with 20 mM Tris. A solution of PsaA protein in 20 mM Tris of pH 8.5 was excited at 280 nm. Emission spectra of the sample was recorded between 290-450 nm. An average of three independent spectroscopic readings was acquired for analyzing the sample.

Characterization by CD spectroscopy

Far UV CD spectra of PsaA was carried out using Jasco-700 Spectro-polarimeter between 190-260 nm wavelength range at 25˚C. Concentrated PsaA solution, incubated in 20 mM Tris were taken in 1mm path length cuvette. An average of three independent spectroscopic readings were acquired for analysis.

4.5 Expression and purification of recombinant SP0845 in *E.coli*

E.coli cells expressing recombinant SP0845 was taken and purification and characterization was performed using the same procedure as described in section 4.4.

5. RESULTS

5.1 Growth kinetics

Growth kinetics of recombinant *E. coli* expression was studied under uninduced and induced conditions. *E.coli* cells harbouring recombinant protein were grown in shaker and induced in mid-log phase when the OD reaches 0.4-0.6 with 1mM IPTG (final concentration) and further grown for 3 hrs. Table 5.1 and figure 5.1 of the growth kinetics relates absorbance of cells at 600 nm to the biomass of the cells. From the figure 5.1 it can be clearly observed that upon induction, they reduce their growth rates considerably and quickly attain stationary phase for the sake of overproduction of protein. The induction of protein was checked by running the uninduced and induced sample on SDS-PAGE gel as shown in the figure 5.2.

Table-5.1: Absorbance of secondary structure of before and after incubation

Time (in hrs)	Induced	Uninduecd
0.5	0.012	0.01
1	0.032	0.034
1.5	0.184	0.174
2	0.321	0.32
2.5	0.657	0.655
3	1.058	0.9
3.5	1.284	1.056
4	1.394	1.194
4.5	1.5	1.32
5	1.561	1.351

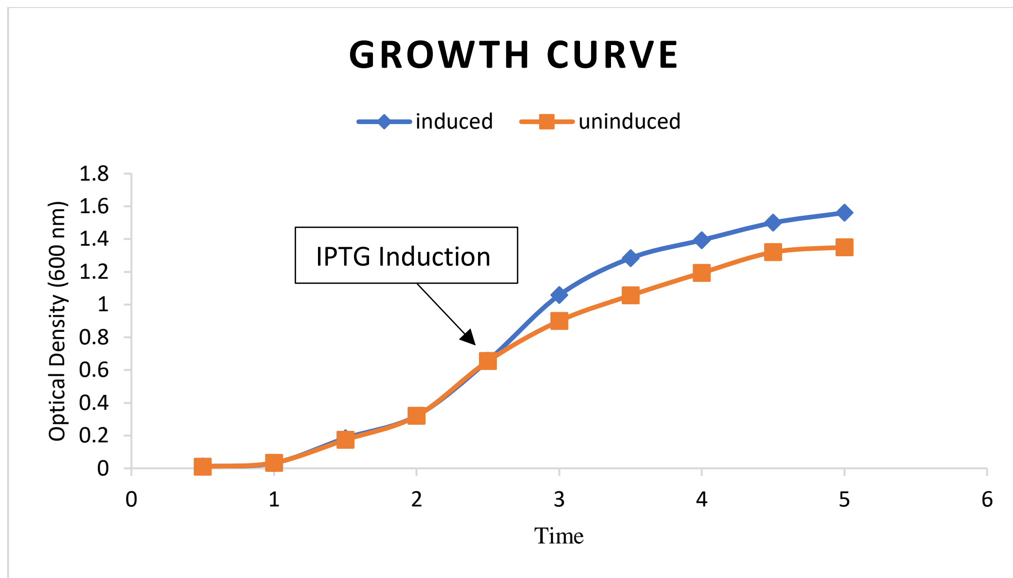


Figure-5.1: Growth kinetics of *E.coli* expressing PsaA

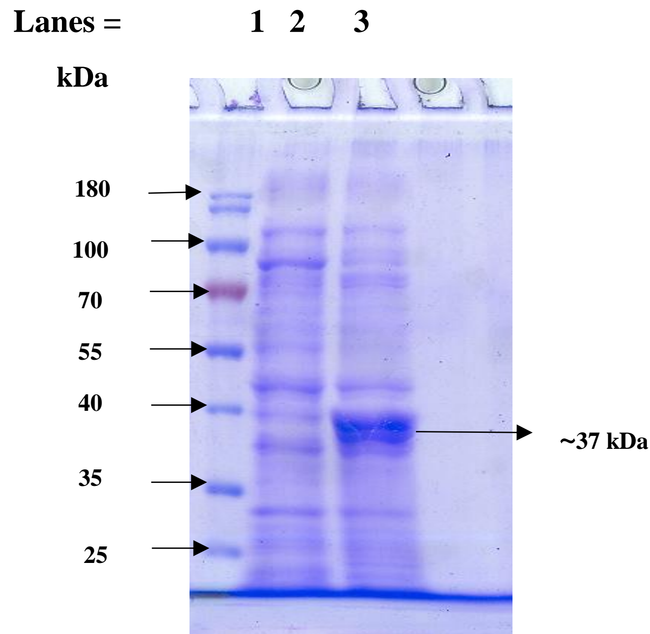


Figure-5.2: Growth curve of *E. coli* expressing recombinant protein in uninduced and induced condition.

Lane 1- Marker, Lane 2- Uninduced, Lane 3- Induced

5.2 Expression and purification of recombinant PsaA in *E.coli*

E.coli cells harbouring recombinant protein were grown in shaker and induced in mid-log phase when the OD reaches 0.4-0.6 with 1mM IPTG. Significant amount of His tagged PsaA were expressed after IPTG induction. After sonication, most of the protein resides was observed in the supernatant. This suggest protein is in soluble form, and was not undergoing aggregation.

After the induction observed cells reduce their growth rate and protein was expressed in induced cells. Protein expression after induction of IPTG was analysed through SDS-PAGE. A sharp band corresponding to apparent molecular weight at ~ 37kDa was observed as shown in figure 5.3.

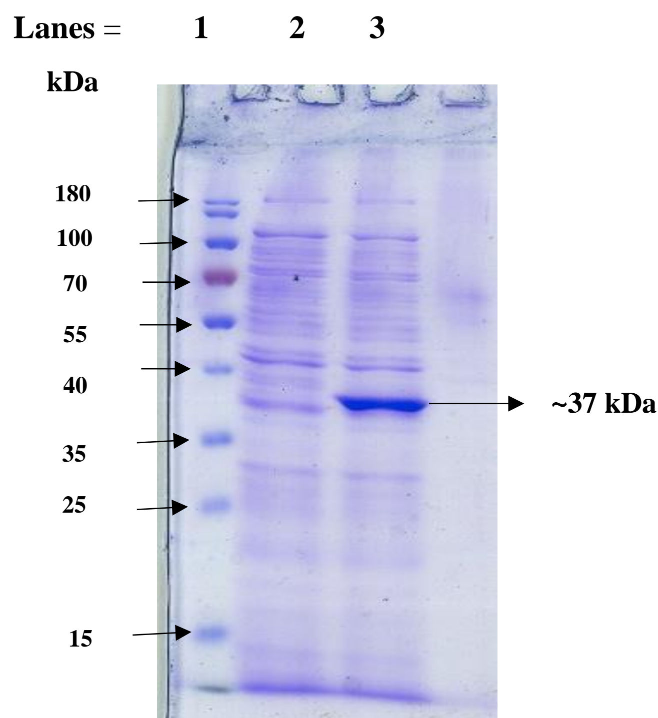


Figure-5.3: PsaA protein expression after IPTG induction

Lane 1- Marker, Lane 2- Uninduced, and Lane 3- Induced

5.2.1 Purification of recombinant PsaA from Ni-NTA chromatography

His tagged protein PsaA was purified by Ni-NTA chromatographic system on Ni-NTA resin. Through the standard immobilization, washing, and elution protocol *E. coli* cells were harvested and centrifuged. Pellet was resuspended and sonicated for cell lysis. The lysed cells were then centrifuged to separate the pellet and supernatant. For his tag protein purification, cell lysed supernatant was loaded onto the matrix. His-tagged proteins were bound, and other proteins pass through the matrix. After washing with the different concentration of imidazole, elutes were analysed by SDS-PAGE. Purified PsaA was observed in 20mM, 50mM, 100mM imidazole elutes as a sharp band corresponding to apparent molecular weight at ~ 37kDa was observed as shown in figure: 5.4

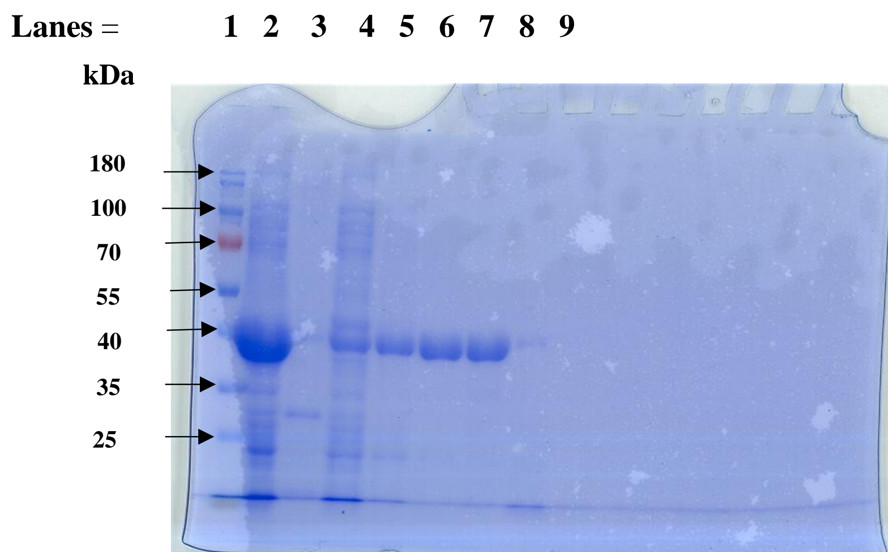


Figure-5.4: SDS analysis of different concentration of imidazole elution of PsaA.

Lane 1- Marker, Lane 2- Load, Lane 3- Flow through, Lane 4- Wash, Lane 5- 20 mM imidazole elute, Lane 6- 50 mM imidazole elute, Lane 7- 100 mM imidazole elute, Lane 8- 250 mM imidazole elute, and Lane 9- 500 mM imidazole elute.

The elute fractions corresponding to 20 mM imidazole concentrate, 50 mM imidazole concentrate, 100 mM imidazole concentrate were pooled and proceeded for desalting followed by lyophilization.

5.2.2 Quantitative estimation of purified PsaA protein by BCA assay

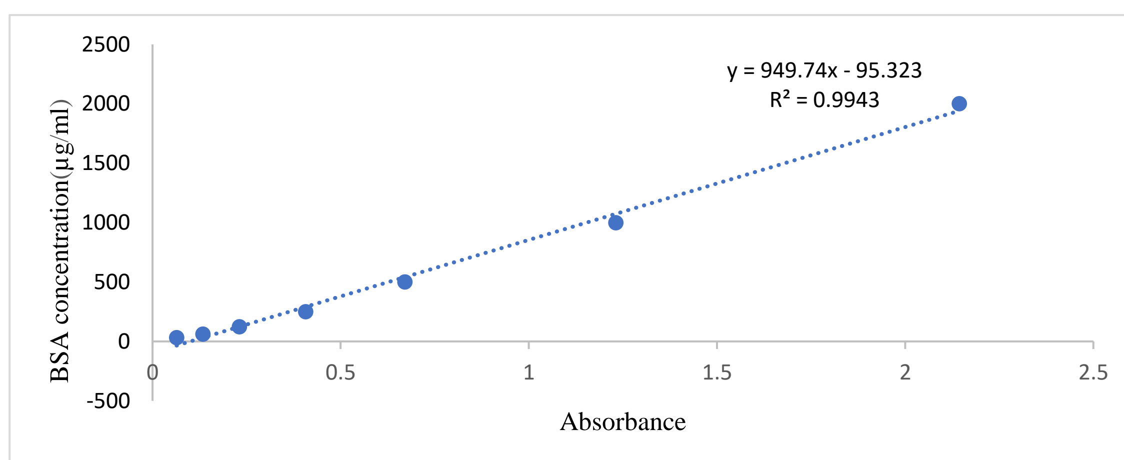


Figure-5.5: Standard graph for BCA protein estimation

About 2.5 mg of lyophilized protein was weighed in an Eppendorf. This was mixed in 500 µL of 20 mM Tris. Upon estimation, the concentration was found to be 1.47

mg/mL. Thus, the actual protein concentration was 30% of the weighed powder. This was then further proceeded for characterization.

5.2.3 Characterization of purified protein

1) Fluorescence spectroscopy

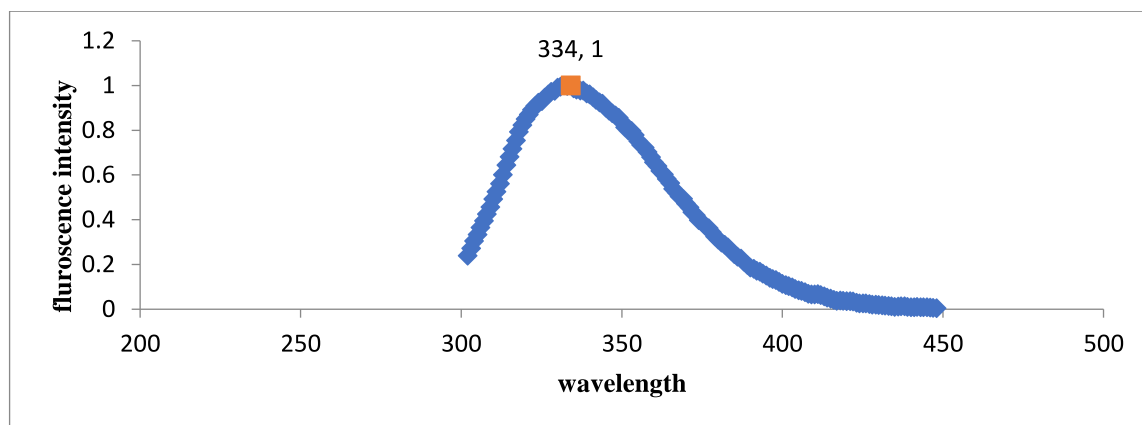


Figure-5.6: Fluorescence spectra of PsaA

Excitation wavelength was 280 nm and the emission spectrum wavelength were from 290-450 nm. From the figure 5.6, it was observed that the PsaA showed the peak at 334 nm. PsaA possess Tyrosine (13), Tryptophan (3) and phenylalanine (11). As we know from the literature the observed peak indicates that these residues possibly occur buried inside the structure of protein.

2) CD spectroscopy

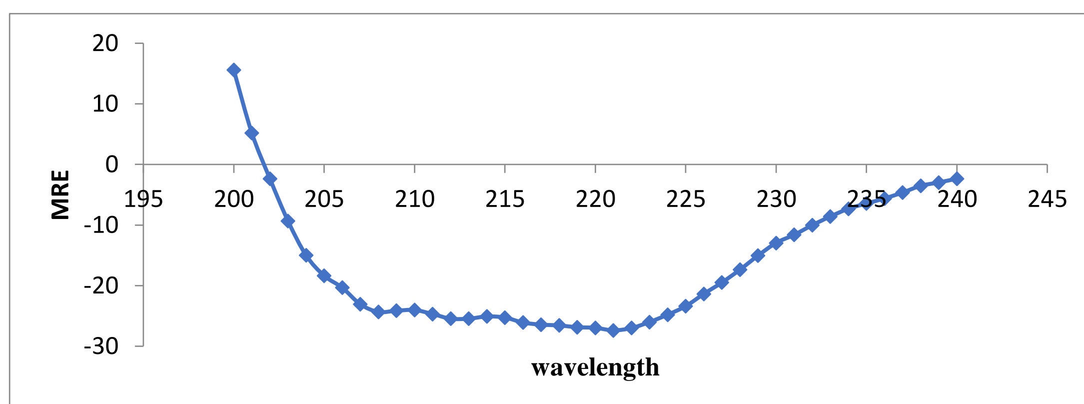


Figure-5.7: CD spectra of PsaA

Alpha helix %= 84.27

Beta strand % = 1.24

Sharp depression at 222 nm and 208 nm are indicative of a majorly alpha helical structure as represented by figure 5.7. Thus, it can be inferred that PsaA exists largely in alpha helical form.

5.3 Expression and purification of recombinant SP0845 in *E.coli*

5.3.1 Purification of recombinant SP0845 from Ni-NTA chromatography

His tagged protein SP0845 was purified by Ni-NTA chromatographic system on Ni-NTA resin. Through standard immobilization, washing, and elution protocol. *E. coli* cells were harvested and centrifuged. Pellet was resuspended and sonicated for cell lysis. The lysed cells were then centrifuged to separate the pellet and supernatant. For his tagged protein purification, cell lysed supernatant was loaded onto the matrix. His-tagged proteins specifically binds to resin with comparatively higher affinity than other proteins. After washing with 20 mM imidazole, elution was carried out with different concentration of imidazole and the elutes were analysed through SDS-PAGE. Purified SP0845 was observed in 50 mM imidazole elute, 100 mM imidazole elute and 250 mM imidazole elute as a sharp band corresponding to apparent molecular weight at ~ 40kDa was observed as shown in figure: 5.8.

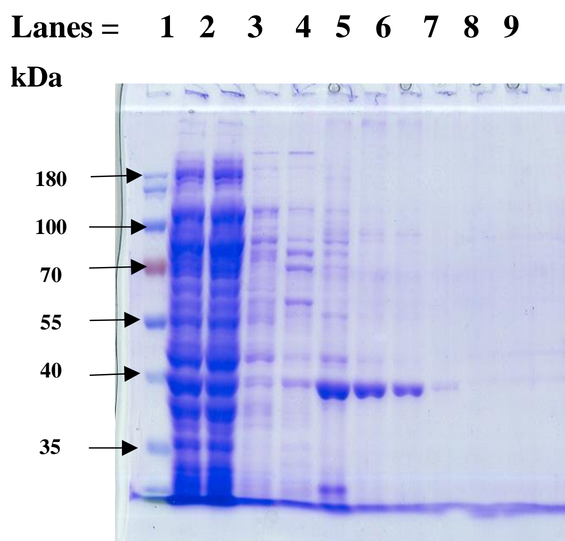


Figure-5.8: SDS analysis of different concentration of imidazole elution of SP0845

Lane 1- Marker, Lane 2- Load, Lane 3- Flow through, Lane 4- Wash, Lane 5- 20 mM imidazole elute, Lane 6- 50 mM imidazole elute, Lane 7- 100 mM imidazole elute, Lane 8- 250 mM imidazole elute, and Lane 9- 500 mM imidazole elute**.**

The elute fractions corresponding to 50 mM, 100 mM, 250 mM imidazole concentrates were pooled and proceeded for desalting followed by lyophilization.

5.3.2 Quantitative estimation of purified SP0845 protein by BCA assay

About 3.2 mg of lyophilized protein was weighed in an Eppendorf. This was dissolved in 500 ul of 20 mM Tris. Upon estimation, the concentration was found to be 2.304 mg/ml. Thus, the actual protein concentration was 36% of the weighed powder. This was then further proceeded for characterization.

5.3.3 Characterization of purified protein

1) Fluorescence spectroscopy

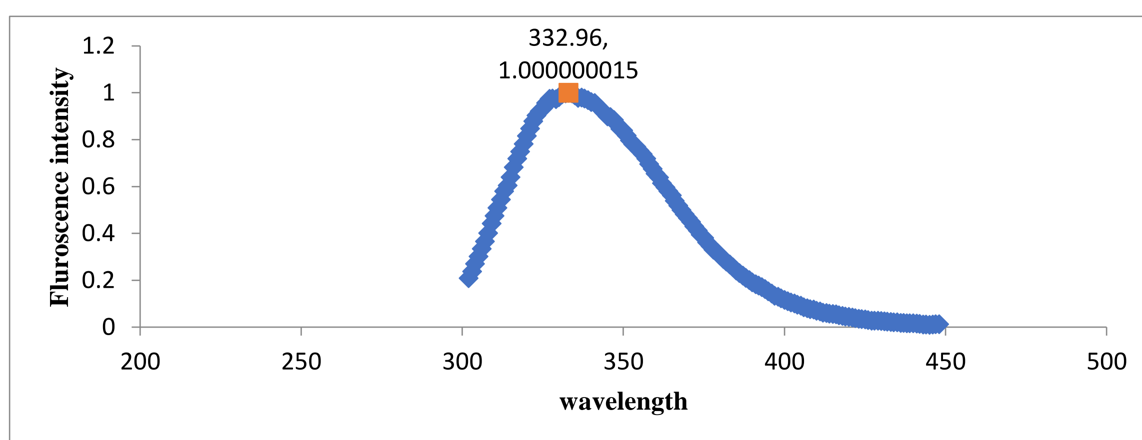


Figure-5.9: Fluorescence spectra of SP0845

Excitation wavelength was 280 nm and the emission spectrum wavelength were from 290-450nm. From the figure 5.9, it was observed that the SP0845 showed the peak at 332 nm. SP0845 possess Tyrosine (10), Tryptophan (4) and phenylalanine (13). As we know from the literature the observed peak indicates that these residues possibly occur buried inside the structure of protein.

2) CD spectroscopy

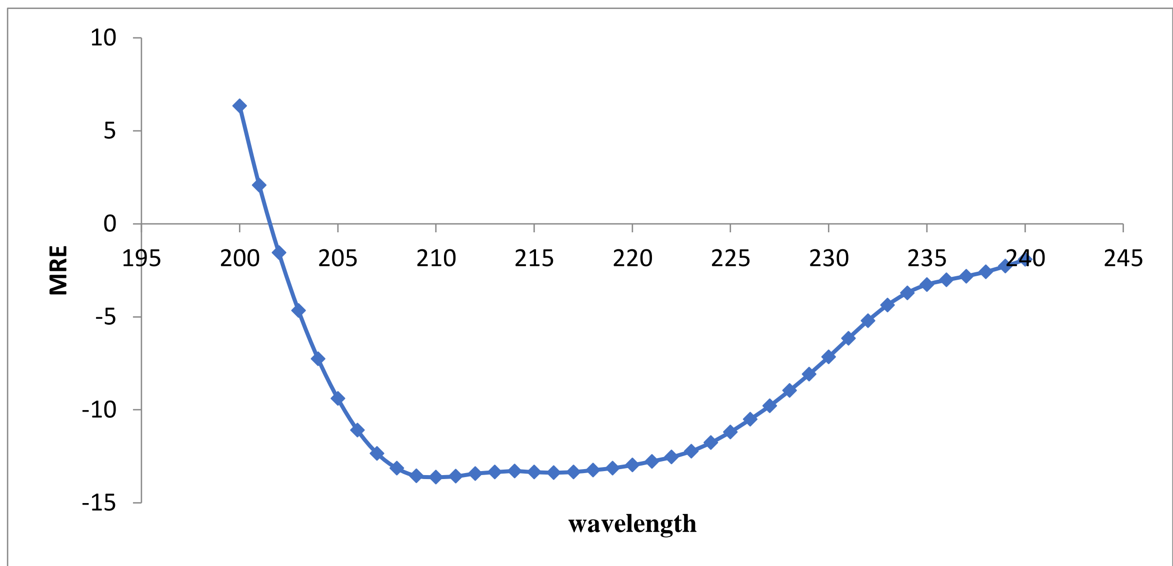


Figure-5.10: CD spectra of SP0845

Alpha helix % = 67.45%

Beta strand % = 3.24

Sharp depression at 222 nm and 208 nm are indicative of a majorly alpha helical structure as represented by figure 5.10. Thus, it can be inferred that SP0845 exists largely in alpha helical form.

6. DISCUSSION

Recent research to look for alternates to the existing polysaccharide vaccines has identified various protein antigens as promising vaccine candidates such as PspA, PsaA, Pneumolysin, SP0845. The aim of this study was to purify pneumococcal surface proteins, PsaA and SP0845, using Ni-NTA chromatography and to characterize them. Pneumococcal surface proteins were induced with IPTG before the purification process. For the purification of induced protein Ni-NTA affinity chromatography was employed as the proteins were tagged with histidine. Purified PsaA was observed in 20mM, 50mM, 100mM imidazole elutes (figure: 5.4) and purified SP0845 was observed in 50mM, 100mM, 250mM imidazole elute (figure:5.5) respectively. The purified fractions were then pooled. The process of desalting was performed as the pooled fractions contained high imidazole content. The percentage purity of protein was estimated which came as expected 30-40%.

Fluroscence spectroscopic analysis showed the maximum fluorescent intensity of PsaA at 334 nm (figure: 5.6) and of SP0845 at 332 nm (figure: 5.9). CD spectroscopic analysis of PsaA and SP0845 showed that both of these proteins have alpha helical structure but further experiments are needed for conclusive evidence as both proteins were his-tagged and his-tags are known to affect the protein.

7. SUMMARY AND CONCLUSION

Streptococcus is a causative agent of meningitis, sepsis, otitis media and pneumonia. Annually, around one million children under the age of 5 years die because of pneumococcal disease. Along with these, resistance to the antibiotics has become a serious issue. Present carbohydrates and conjugated vaccine have the drawback that it has very limited affect in children, little effect in elderly and also less potent in patient with immunodeficiency. In this work an attempt has been made to produce protein-based vaccine for pneumonia which would overcome the drawback of currently available carbohydrate and conjugate vaccine.

Protein vaccines are less complicated and possibly cheaper than Polysaccharide conjugate vaccines. Protein based vaccines provide the broad serotype coverage than the polysaccharide and conjugate vaccines.

To overcome the disadvantages of serotype-specific vaccines, a number of pneumococcus proteins are being studied as vaccine candidates. These proteins include pneumolysin, pneumococcal surface protein A (PspA), and pneumococcal surface adhesin A (PsaA). Most proteins induce a certain level of defence against pneumococcus carriage and invasive disease in mice, their protective capacities in humans remain uncertain.

PsaA is the most common protein in *Streptococcus pneumoniae* which is conserved on all the pneumococcal serotypes across the boundaries. Studies have shown the immune compatibility of pneumococcal surface adhesin A with other Pneumococcal proteins. It can be considered as one of the vaccine candidates as it is the major virulence element which facilitates pneumococcal nasopharyngeal colonization.

We chose PsaA and SP0845 highly conserved proteins. They were successfully expressed in *E.coli* bacterial system. Further, we were able to purify the proteins via Ni-NTA affinity chromatography with appreciable purity. Fluorescence spectroscopy and circular dichroism analysis were done to characterize them. The secondary structure studies predicted that both the proteins PsaA and SP0845 existed in the alpha helical form.

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9. APPENDIX

Techniques used purification and characterization of protein

A. Affinity Chromatography (Ni-NTA)

Affinity Chromatography is a purification system that enables the purification of biomolecules with respect to Individual chemical structure or biological function. During the process of purification, the sample gets specifically and reversibly bind to the binding agent called ligand, which is immobilized to the matrix through covalent bonds. Samples are provided under favourable circumstances that specifically bind to the ligand. As a result, while the compound is not washed away, the substance is preferred connected to the ligand. By changing the empirical conditions to facilitate absorption, recovery of desired molecules can be achieved through washing and cleaning.

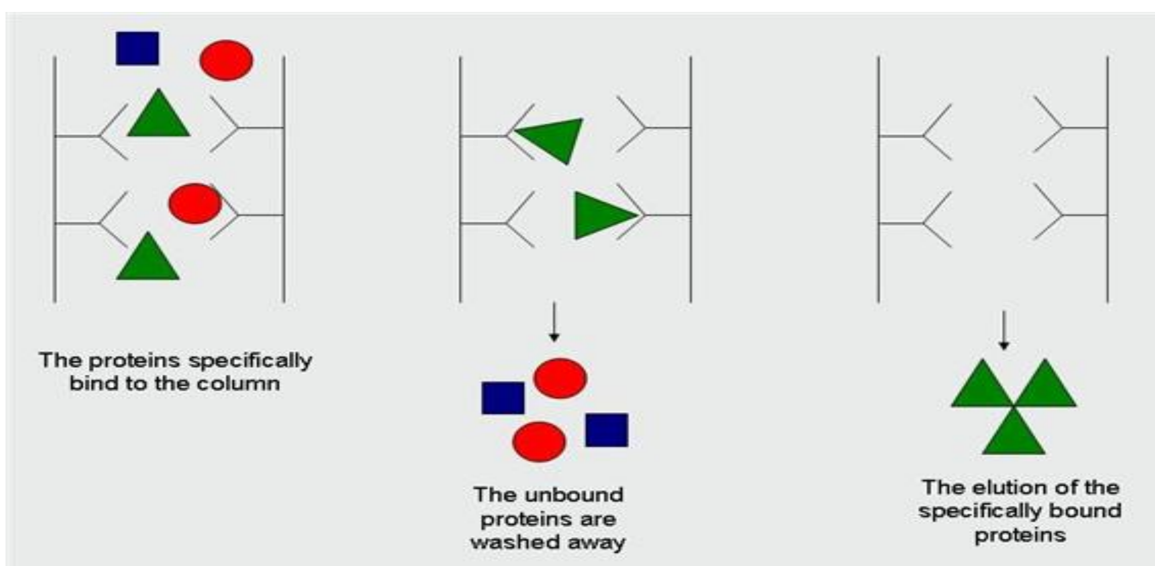

Figure-9.1: Purification of protein from affinity chromatography (adapted form Hage et al 2005)

The Ni-NTA related chromatography is designed to purify the 6xHis recombinant proteins expressed in bacterial cells, insect cells and mammalian cells. The chromatographic system is intended for elevated selectivity and affinity of Ni-NTA agarose for recombinant proteins, which are labelled with 6x his residues. Ni-NTA

chromatographic system consists of buffers and resins to sterilize proteins in denaturing, native or hybrid conditions. Ni-NTA system uses nitriloacetic acid, a tetradentate chelating agent treated in 6% of polar cross-bridge matrix. NTA combines Ni^{2+} plus ions with four coordination bonds. Resin-bound proteins are eluted by imidazole or pH change.

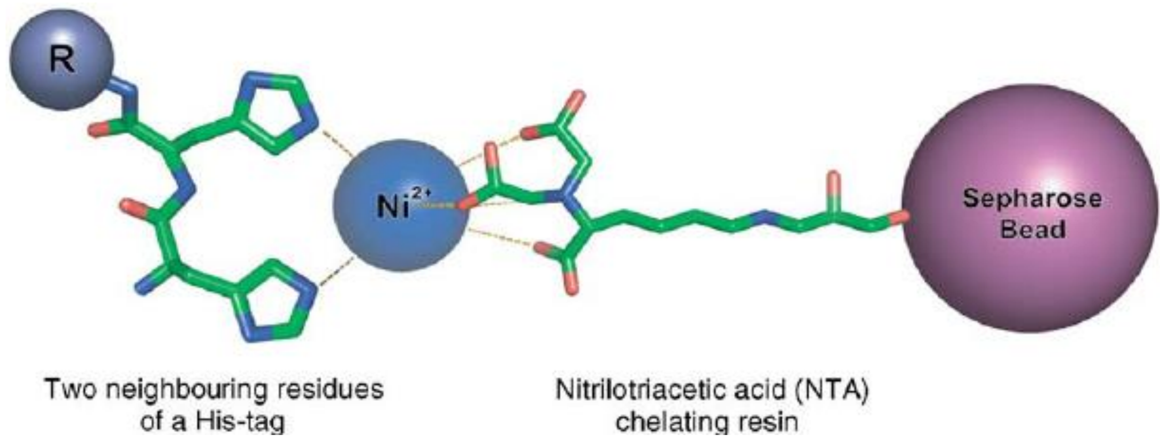

Figure-9.2: Mechanism of Binding of Histidine (adapted from Bolanos-Garcia et al. 2006)

B. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SDS-PAGE is an electrophoresis system used for the separation charged molecules in a mixture based upon their molecular weight in an applied electric field. It is a discontinuous electrophoresis method mainly used for the separation of proteins with molecular weight from 5 to 250kDa. The system was invented by Ulrich K. Laemmli. The system typically consists of two gels stacks-the lower one is resolving gel and stacking gel laid over it. The proteins get concentrated in the stacking before entering the resolving gel whereas, they get separated on the basis of their molecular weights in resolving gel. The composition difference of resolving gel, stacking gel, and the electrophoretic buffer forms a system which capable to resolve the proteins based upon their molecular weights.

Electric field is generally applied to the electrophoretic chamber for the movement of charged molecules through polymerized gel called polyacrylamide gel. The charged molecules move through the gel matrix due to its porous structure. It is the diameter of the pore and shape and size of the molecule based upon which the gel matrix provides

resistance to the individual molecule. However, to control the size of pore, the gel monomer concentration could be adjusted within a defined range. Mainly, the molecules with small size bearing high charge moves faster through the gel matrix rather than the molecules with molecules with large size and less charge.

Acrylamide and N,N'methylenebisacrylamide (a cross linking agent) together forms the polyacrylamide gel, used for the separation of protein molecules It is the concentration of the acrylamide, as well as the ratio of acrylamide to bisacrylamide that controls the size of the pore in the gel. When ammonium persulfate, a catalyst, reacts with another catalyst, TEMED, free oxygen radicals are generated. When these free oxygen radicals react to vinyl group of acrylamide and bsacrylamide, process of polymerization occurs.

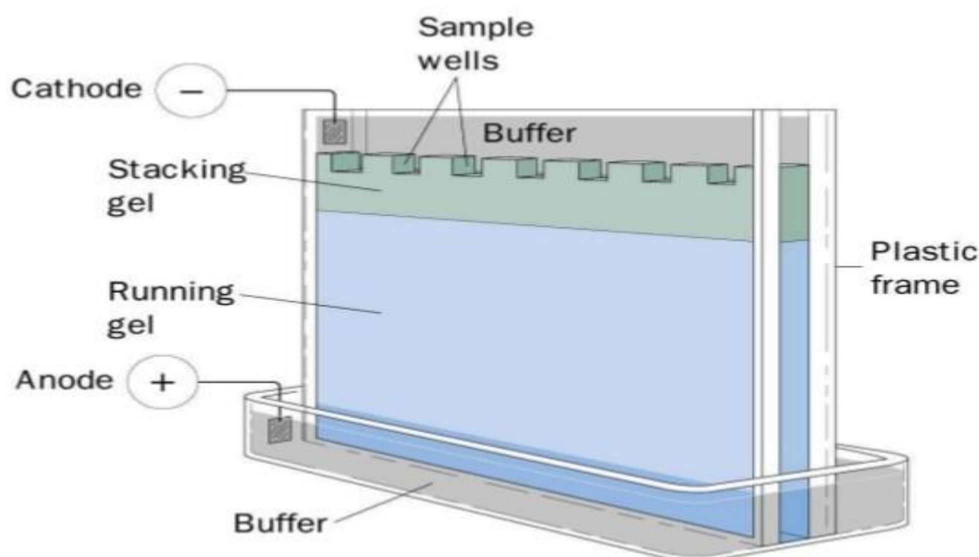

Figure-9.3: Apparatus for SDS-PAGE (adapted from Hartfelder et al. 2013).

C. Circular dichroism spectroscopy

Circular dichroism spectroscopy is a technique in which the Circular dichroism of the molecules is measured over a range of wavelengths. For the study of chiral compounds of varying size and type, CD spectroscopy is extensively used. CD spectroscopy is a type of absorption spectroscopy which measures the difference in absorbance of right circular polarized light and left circular polarised light by a substance. It is frequently used to study macromolecules secondary structure or conformation, especially proteins. The secondary structure of proteins is sensitive to pH, temperature, environmental conditions therefore CD spectroscopy is used to analyse the change in proteins

secondary structure with respect to environmental factors or when the protein molecules interact with each other. The CD spectra between 180nm to 260nm is studied for the analyses of secondary structure of proteins like α-helical structure, beta pleated sheets, turn and other.

Most of the biomolecules shows the chiral property. 19 out of the 20 amino acids that are formation units of proteins are even chiral, so as various other molecules along with the highly structural proteins, DNA and RNA. Circular dichroism is well suited to study the highly chiral chemistry of molecules and the analysis of biomolecules is the primary application of CD spectroscopy.

Circular dichroism spectroscopy is mainly used to compare two biological molecules or the similar compound under different conditions and analyse whether they have alike structure or not. The system can be used to determine if a newly purified protein is properly folded, determining whether a mutant protein has a similar folding pattern compared to wild type or to analyse biopharmaceutical products to confirm that they are still in a properly folded active conformation.

Instrumentation

The CD instruments are based on the technique introduced by Grosjean and Legrand. The unpolarized light from the light source passes through the monochromator. The polarized light then passes through a modulator known as photo elastic modulator (PEM), which functions by converting linear light to circularly polarized light. The incident light on the sample switches between LCP and RCP light. As the incident light, switches direction of polarization the absorption changes and the differential molar absorptivity can be calculated.

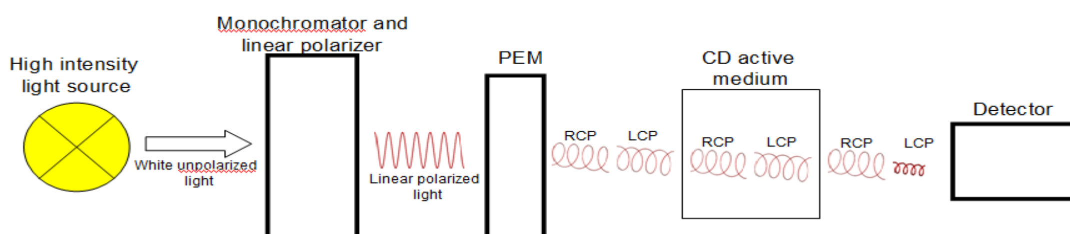

Figure-9.4: The instrumentation for CD spectroscopy (adapted from Greenfield 2006).

D. Fluorescence spectroscopy

Fluorescence spectroscopy is a technique that speculates fluorescence in a sample. It involves the use of light rays, usually ultraviolet rays to stimulate electrons in certain molecular compounds to emit light. Aromatic compounds such as tryptophan, tyrosine and phenylalanine absorb ultraviolet light. The aromatic chains of this amino acid are responsible for UV absorption. Tryptophan and Tyrosine absorb up to 280 nm. However, phenyl alanine absorbs up to 257.4 nm. Absorption of 280 nm is used for the detection and quantification of purified proteins. The rate of absorption of each protein depends on the number and location of amino acid residues in aromatic acids.

Molecules have several states called energy levels. Fluorescence spectroscopy includes electronic and vibration states. Basically, the species under study has a ground state or a low energy state and high-energy stimulating state called excited state. In this, each electronic state is imposed of a different vibration state. In fluorescence, the species under study absorbs the photon of light, and gets excited from ground state to one of the various vibrational state in excited state. As the molecules collide with each other, the excited molecules keep losing the vibrational energy till they reach the lowest vibrational level in the excited state. The process is usually represented through jablosnski diagram.

The excited molecule emits photon as it falls down to the vibrational state of the ground state. The emitted photon will have different energy so as the frequency as the molecule falls down to the vibrational state of ground state. Hence, the structure of various vibrational states can be analysed by determining the different frequencies of emitted light in fluorescent spectroscopy, together with their relative intensities.

Instrumentation

All fluorescent instruments are composed of three basic elements: -light source (excitation source), sample holder and detectors. For analysis and use, the wavelength of the radiant light must be selected and the detector signal must be effective enough to manipulate it and accurately display it. In a fluorescence system, the wavelength of excited and emitted light is selected by the filter, enabling measurement made at any pair of fixed wavelengths.

As the light from the light source flows through the monochromator, it hits the sample. The molecules in the sample absorbs a portion of this incident light and fluoresce. The fluorescent light is scattered in every direction. As the light flows from the second monochromator, some of the light reaches to the detector. The detector is generally placed at 90˚to the incident ray of light. The detector records the signal and amplify it and gives the reading.

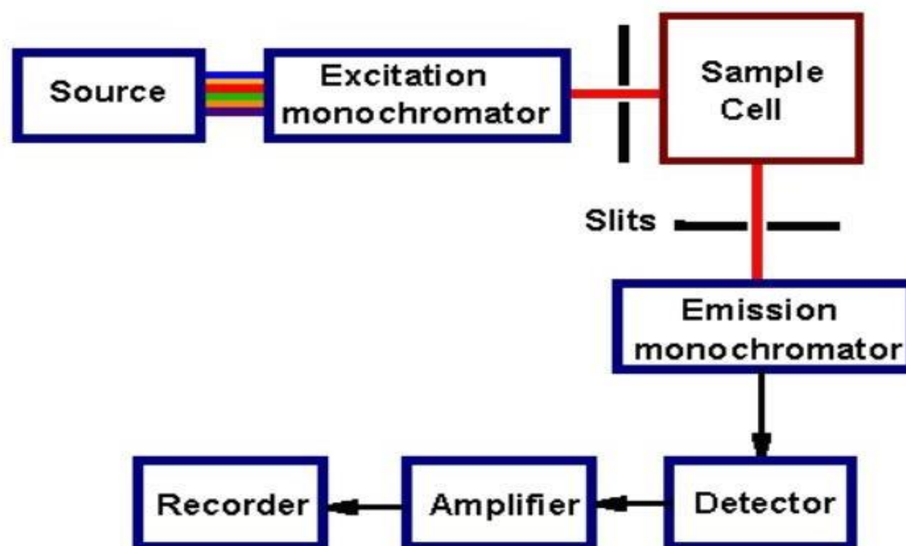

Figure-9.5: Components of Fluorescence spectrophotometer (adapted from Bachmann et al. 2006)

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