Expression and Functional Characterization of putative TatD Family Hydrolase and Haemolysin III of *Clostridium chauvoei*

Thesis

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Master of Veterinary Science
(Veterinary Bacteriology)

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Dedicated to...

My Beloved Family

भा.कृ.अनु.प.–भारतीय पशु चिकित्सा अनुसंधान संस्थान
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Dated: 04/07/2017

Certificate

This is to be certified that the research work embodied in this thesis entitled **"Expression and functional characterization of putative TatD family hydrolase and Haemolysin III of *Clostridium chauvoei*"** *submitted by* **Dr. Aakanksha Tiwari, Roll No. M-5603,** *for the award of* **Master of Veterinary Science** *Degree in* **Veterinary Bacteriology** *at ICAR-Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate herself under my supervision and guidance.*

It is further certified that **Dr. Aakanksha Tiwari, Roll No. M-5603,** *has worked for more than 21 months in the Institute and has put in more than 150 days attendance under me from the date of registration for the* **Master of Veterinary Science** *Degree in this Deemed University, as required under the relevant ordinance.*

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We the undersigned members of Advisory Committee of **Dr. Aakanksha Tiwari, Roll No. M-5603,** *a candidate for the degree of* **Master of Veterinary Science** *with the major discipline in* **Veterinary Bacteriology,** *agree that the thesis entitled* "**Expression and functional characterization of putative TatD family hydrolase and Haemolysin III of *Clostridium chauvoei***" *may be submitted in partial fulfilment of the requirement for the degree.*

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Master of Veterinary Science** *Degree of this Institute.*

It is further certified that the candidate has completed all the prescribed requirements governing the award of **Master of Veterinary Science** *Degree of the Deemed University, Indian Veterinary Research Institute, Izatnagar.*

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(Aakanksha Tiwari)

ABBREVIATIONS

%	:	Percentage
/	:	per
@	:	At the rate of
6X	:	6 times
APS	:	Ammonium per sulphate
ATCC	:	American Type Culture Collection
bp	:	Base pairs
BSA	:	Bovine serum albumin
C. chauvoei	:	*Clostridium chauvoei*
$CaCl_2$	:	Calcium Chloride
CctA	:	*Clostridium chauvoei* toxin A
DAB	:	Diaminobenzidine
DNA	:	Deoxyribo nucleic acid
dNTPs	:	Deoxyribonucleotide triphosphate
E. cloni	:	*Escherichia cloni*
EDTA	:	Ethylenediamine tetra acetic acid
et al.	:	and co workers
Fig.	:	Figure
g	:	Gram
GDP	:	Gross Domestic Product
H_2O_2	:	Hydrogen peroxide
HCl	:	Hydrochloric acid
His	:	Histidine
Hr(s)	:	Hour(s)
HRPO	:	Horse radish peroxidase
Ig	:	Immunoglobulin
IgY HRPO	:	Immunoglobulin Y Horse radish peroxidase
IPTG	:	Isopropyl β-D-1-thiogalactopyranoside
Kb	:	Kilo base pair
KCl	:	Potassium Chloride
kDa	:	Kilodalton
KH_2PO_4	:	Potassium dihydrogen phosphate
LB	:	Luria Bertani
M	:	Molar
mg	:	Milligram(s)
$MgCl_2$	:	Magnesium Chloride
Min	:	Minutes

ml	:	Millilitre
mM	:	Millimolar
Mol. wt.	:	Molecular weight
Na_2HPO_4	:	Di sodium hydrogen phosphate
NaCl	:	Sodium Chloride
NaH_2PO_4	:	Sodium dihydrogen phosphate
$NaHCO_3$	:	Sodium bicarbonate
NCBI	:	*National Center for Biotechnology Information*
NCM	:	Nitrocellulose membrane
NFW	:	Nuclease free water
nts	:	Nucleotides
^oC	:	Degree centigrade
OD	:	Optical density
OPD	:	Orthophenylene diamine
PBS	:	Phosphate buffered saline
PBST	:	Phosphate buffered saline with 0.5%Tween 20
PCR	:	Polymerase Chain Reaction
PEG	:	Polyethylene Glycol
pH	:	Log hydrogen ion concentration
pmoles	:	Pico moles
RE	:	Restriction enzyme
RNA	:	Ribonucleic acid
rpm	:	Rotations per minute
S	:	Svedberg unit
SDS-PAGE	:	Sodium dodecyl sulphate Polyacrylamide gel electrophoresis
sec	:	Seconds
sp.	:	species
Ta	:	Annealing temperature
TAE	:	Tris acetate ethylelediamine tetra acetic acid
TEMED	:	N, N, N', N' – Tetra methyl ethylene diamine
TSS	:	Transformation and Storage Solution
USA	:	United States of America
V	:	Volts
v/v	:	Volume/volume
w/v	:	Weight/volume
µg	:	Microgram
µl	:	Microlitre
µM	:	Micro molar

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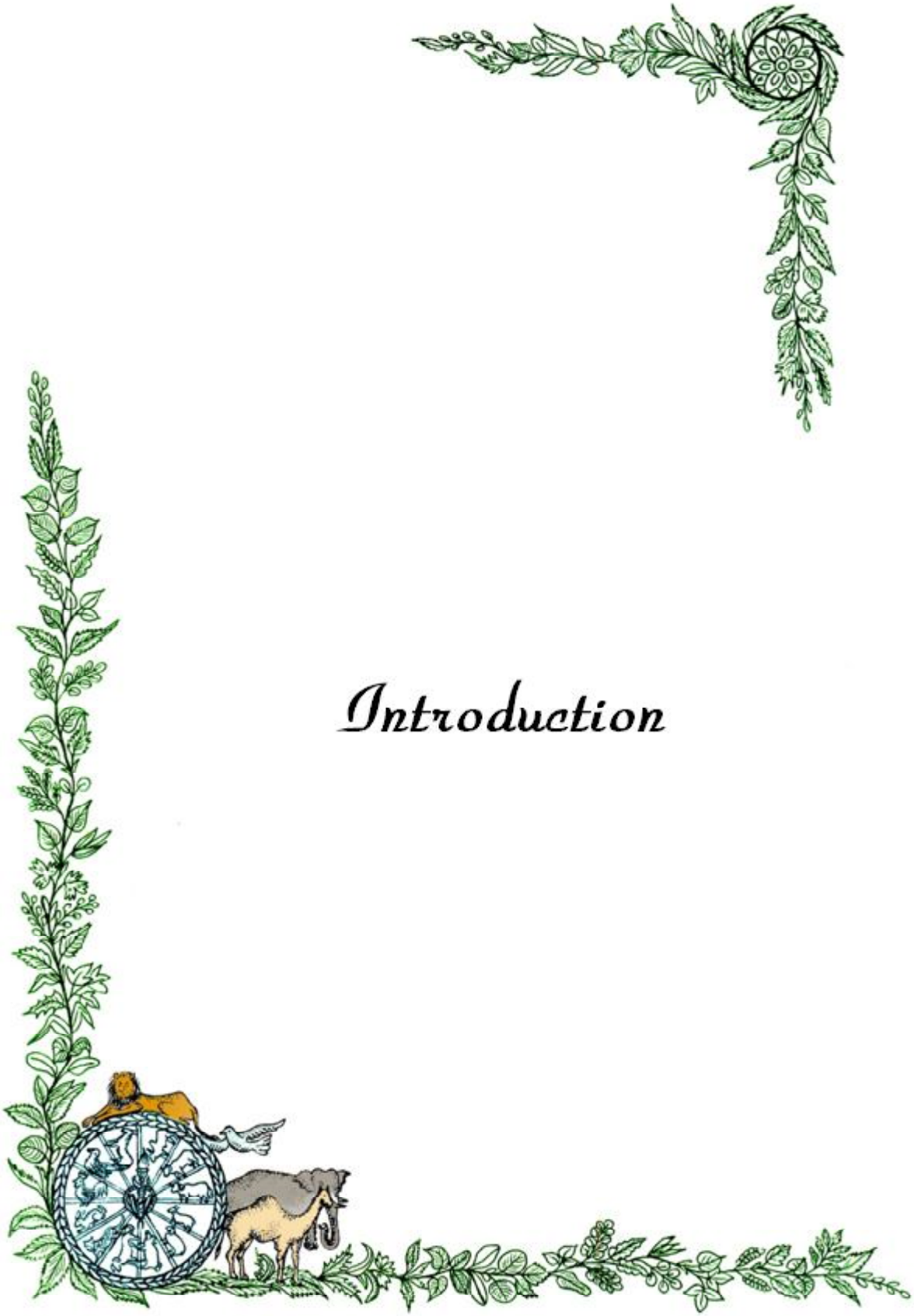


Introduction

1 INTRODUCTION

Livestock production contributes significantly to the agricultural economy of the developing countries. It has a contribution that goes beyond direct food production to include multipurpose uses. In 2010-11 livestock generated outputs worth Rs 2075 billion (at 2004-05 prices) which comprised 4% of the GDP and 26% of the agricultural GDP (Tabrez and Khan, 2008). The total output worth was higher than the value of food grains. However, there are many deadly pathogens which have affected the livestock immensely and led to serious consequences. These include Bovine Respiratory Disease Complex, Blackleg, Bovine Respiratory Syncytial Virus, Bovine Viral Diarrhoea, *Haemophilus somnus*, Infectious Bovine Rhinotracheitis, Parainfluenza Type 3 and Haemorrhagic septicemia.

Blackleg or Black Quarter is an acute, febrile and highly fatal disease of cattle and sheep caused by *Clostridium chauvoei* and characterized by emphysematous swelling, commonly affecting heavy muscles (Clostridial myositis). Most cases are seen in cattle of 6–24 months age, but thrifty calves as young as 6 wk and cattle as old as 10–12 yr may also be affected. Black quarter is a soil-borne infection mostly occurring in rainy season. Disease is widespread in Karnataka, Tamil Nadu, Andhra Pradesh, Maharashtra and coastal region and sporadically occurs in Northern and Eastern states of India. In recent times, maximum outbreaks occurred in year 2009-2010 in West Bengal with total of 95 outbreaks, affecting 293 animals with 122 deaths. In the same year in Maharashtra, there were 37 outbreaks attacking 128 heads with 96 deaths. Trends indicated gradual decrease in disease occurring from 2006-2010 [Anonymous, 2011]. In the year 2010 at national level, there were 369 outbreaks, 4707 attacks and 514 deaths due to Black Quarter (Annual Report DADF, 2011-12). The

name 'blackleg' derives from the fact that the site of infection is often a leg muscle, and that the affected muscle is dark in colour. It produces an acute local infection. In the beginning of infection, animals may have fever and affected limb is hot to touch, limb usually swells and animal develops lameness on that leg. Crepitation sound is heard when the affected portion of the limb is pressed. Infected animal dies within 12 to 36 hrs after the appearance of the first symptoms [Hatheway, 1990].

Blackleg is caused by *Clostridium chauvoei,* a Gram positive, anaerobic bacilli. It is found naturally in the intestinal tract of animals. Spores remain viable in the soil for years and are supposed to be a source of infection. They remain in the soil in an inactive state, and return to their infectious form when consumed by grazing livestock. The infection begins when the susceptible animal ingests the endospores which then crosses the gastrointestinal tract and enter the bloodstream. Endospores are deposited especially in heavy muscles. They lie dormant in the tissue until they become activated and stimulate the infection [Sathish and Swaminathan, 2008]. Conditions such as bruising of muscle helps these organisms to begin their multiplication, produce toxins and other antigenic components that may be highly fatal [Richey, 2004].

It has been postulated that *C. chauvoei* produces four major toxins, viz., oxygen stable haemolysin (alpha toxin), deoxyribonuclease (beta toxin), hyaluronidase (gamma toxin) and oxygen-labile haemolysin (delta toxin) as well as sialidase and flagellin [Smith and Williams, 1984; Tamura *et al*., 1995], which are proposed to make major contribution to the pathogenicity of *C. chauvoei.* However, among these proposed toxins, the existence of genes for sialidase and flagellin only have been proved [Tamura *et al*.,1984; 1995, Vilei *et al*.,2011], and the role of other toxins, viz., alpha, beta, gamma and delta toxins and the existence of genes encoding these toxins was not known.

Recently, whole genome of *C. chauvoei* has been sequenced [Frey and Falquet, 2014] and the complete genome is available at NCBI website (NCBI Accession No. NZ_LT799839.1). Though it has been proposed that *C. chauvoei* produces deoxyribonuclease and its enzymatic activity was made evident [Carloni *et al.*, 2005], whole genome sequence of *C. chauvoei* did not reveal any specific gene for deoxyribonuclease. However, homology search of the *C. chauvoei* genome sequence using known deoxyribonuclease genes from

other *Clostridium* species such as *C. difficile* and *C. butyricum* has shown significant similarity to the TatD hydrolase (*tatD*) gene of *C. chauvoei*. TatD hydrolase is a cytoplasmic protein, expressed by various bacterial species, having magnesium dependent deoxyribonuclease activity [Chen *et al*., 2014]. *tatD* is detected almost exclusively in the cytoplasmic protein fraction in *E. coli* strongly suggesting that it is normally located in the cytoplasm [Wexler *et al*., 2000]. In *C. elegans, tatD* homologue CRN-2, was shown to have DNA fragmentation activity [Parrish and Ding, 2003]. Hence, we hypothesized that TatD hydrolase of *C. chauvoei* could be putative deoxyribonuclease.

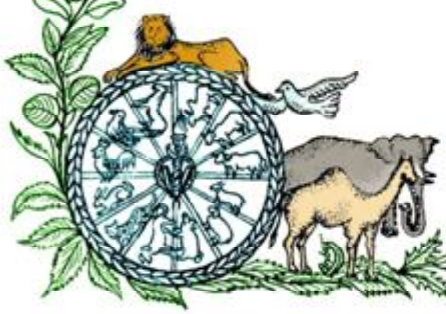
Genes for haemolysins produced by *C. chauvoei* viz., *Clostridium chauvoei* toxin A (*cctA*), Haemolysin A, Haemolysin XhlA and Haemolysin III (*hlyIII*) have been annotated [Frey and Falquet, 2014]. Among these, *cctA* has been shown to be major haemolysin and cytotoxin [Frey *et al.*, 2012]. Role of Haemolysin A in the pathogenesis of *C. chauvoei* infections is uncertain and its haemolytic function could not be verified [Frey and Falquet, 2014]. Haemolysin XhlA is a small protein of only 8.8 kDa size, present mostly in spore forming environmental bacterium and its role in pathogenesis of blackleg disease is questionable [Frey and Falquet, 2014]. However, Haemolysin III is a 25 kDa protein, highly similar to Haemolysin D of *C. perfringens* and could represent 27 kDa protein that was identified in *C. chauvoei* strain C6H [Hang'ombe *et al*., 2000]. It is a pore-forming haemolysin with functional pore diameter of about 3-3.5 nm and is also shown to be present in most of the pathogenic bacteria including *Bacillus* species, where it is named as one of the haemolysin toxins [Baida and Kuzmin, 1996]. We hypothesize that though *cctA* is a major haemolysin of *C. chauvoei, hlyIII* may also play a role as a haemolysin.

Given this, to characterize the functional aspects of TatD hydrolase and Haemolysin III of *C. chauvoei* in order to better understand their role in the pathogenesis of *C. chauvoei*, the present study was undertaken with the following objectives:

(i) To clone and express *tatD* and *hlyIII* genes of *C. chauvoei* .

(ii) Functional characterization of the expressed TatD hydrolase and Haemolysin III genes.

Review of Literature



2 REVIEW OF LITERATURE

Ruminant livestock play a very major role in national economy and hence their care and proper livelihood is of foremost importance. There are various diseases which affect our livestock severely and leads to fatal outcomes. One of these diseases, one important disease is Blackleg, a fatal disease leading to the death of the animal within 12-36 hrs. It is mainly seen in cattle and sometimes also in sheep. It is a severe disease with heavy mortality.

2.1 BLACKLEG

In cattle, blackleg infection is endogenous. Lesions develop without any history of wounds, although bruising or excessive exercise may precipitate disease in some cases. Commonly, the animals that contract blackleg are of the beef breeds, in excellent health, and gaining weight. Most cases are seen in cattle from 6–24 months old, but thrifty calves as young as 6 wk and cattle as old as 10–12 yr may also be affected. The disease usually occurs in summer and rainy season and is uncommon during the winter. In sheep, the disease is almost always the result of a wound infection and often follows some form of injury such as shearing cuts, docking or castration. Blackleg is also known as Black quarter, Quarter evil, Quarter ill, Rauschbrand, Gerausch and Charbon symptomatique. This disease was for the first time reported by Bollinger in 1875. He studied the tissues of dying cattle and observed microscopically a number of short bacteria. He also reported that these bacteria were different from the anthrax bacillus [Scott, 1924]. It is an economically imporant disease amongst the marginal dairy farmers of India though preventable by vaccination, occurs sporadically.

2.2 ETIOLOGICAL AGENT

Blackleg is caused by *Clostridium chauvoei*, a Gram-positive, spore-forming, rod-shaped, histotoxic anaerobe that has strong hemolytic activity. It is difficult to isolate because of its vigorous motility and tendency to swarm on the surface of agar media. [Willis, 1969]. The cell dimensions of *Clostridium chauvoei* are 0.5-1.7 µm breadth and 1.6-9.7 µm in length. It is grouped under 16S rRNA gene cluster I. *C. chauvoei* is one of the most pathogenic species in genus *Clostridium*. Bollinger reported in 1875 that numerous short bacteria observed microscopically in tissues from cattle dying from Gerausch had no similarity whatsoever with the filamentous anthrax bacillus. Arloing and co workers described the blackleg organism in some detail in 1880 [Niles, 1897]. The name *Clostridium chauvoei* was given in honour of the French veterinarian Auguste Chauveau.

The genus *Clostridium* consists of organisms whose G+C contents vary from 22 to 55 mol% [Hippe *et al*., 1991]. The spores of the bacterium are very resistant to adverse environmental stress. The spores can withstand high temperature (120°C for 10 min) and dessication and are resistant to disinfectants. The organism can be destroyed by 3% formalin in 15 min and by 2% bichloride of mercury in 10 min. In soil, the spores can persist for a number of years. *C. chauvoei* and *C. septicum* are difficult to distinguish from each other on the basis of their physiological and toxigenic characteristics. Both are rather oxygen tolerant and easy to culture. Both organisms ferment glucose, fructose, lactose, maltose and mannose. They liquefy gelatin but do not digest meat or milk proteins. Both produce acetic and butyric acids as metabolic end products. A differential characteristic that is widely accepted is sucrose fermentation, which is positive for *C. chauvoei* and negative for *C. septicum* [Cato *et al*., 1986; Holdeman *et al*., 1977], but it has been reported that this distinction is unreliable [Al-Khatib, 1969]. It has also been reported that long chains of bacilli or long filaments in the serous cavities and on the liver surface in infected animals are formed by *C. septicum* but not by *C. chauvoei* [Willis, 1969; Heller, 1920]. *C. chauvoei* does not ferment salicin unlike *C. septicum*. Indole is not produced by it. It is MR and VP positive; does not reduce nitrate and also does not produce hydrogen sulphide. Catalase is also not formed.

2.3 GROWTH CHARACTERISTICS

C. chauvoei is a little demanding, it does not grow on simple nutrient media rather growth is improved by addition of liver extract and glucose. In Robertson cooked meat medium, growth is little slow but very well. The meat particle turns red i.e. the bacteria is saccharolytic in nature and turbidity produced is little. Growth gives a typical rancid, acidic smell due to production of butyric acid. Blood agar containing liver extract will support the growth in which whitish-grey colonies with beta haemolysis is observed (2-4 mm diameter). These organisms are strict anaerobes. They appear either singly or in short chains of 3-4. Their endospores are usually oval and can distort the morphology of the bacterial cell forming shapes like - citron shape or cigar shape.

2.4 EPIDEMIOLOGY

Black quarter (BQ), the second most important disease with morbidity, mortality, temporal and spatial patterns varied considerably. BQ forecasting model has also been given for India which recognised the true occurrences at 76.21 % for BQ. In contrast, the true non-occurrences were 78.18 % . The sensitivity and specificity were 71.39 and 82.15 for BQ. Interestingly, the proportion of false positives ranged from 12.5 to 17.8% and false negatives ranged from 28.60 to 38.94% [Sudhindhra, 1997]. Disease is widespread in Karnataka, Tamil Nadu, Andhra Pradesh and Maharashtra. Maximum outbreaks occur in year 2009-2010 in West Bengal with total of 95 outbreaks, affecting 293 animals with 122 deaths. In the same year in Maharashtra 37 outbreaks attacking 128 heads with 96 deaths. The majority of black quarter cases occur in young cattle of 3 months to 2 years of age. Spores of *C. chauvoei* are capable of surviving for many years in soil. Pasture contamination with spores leads to frequent outbreaks in endemic areas. According to the report by Ministry of Animal husbandry, Fisheries and Dairying, Government of India, there were 68 outbreaks, 547 attacks and 218 deaths in India due to BQ from January 2016 to October 2016 alone in cattle, buffaloes sheep and goats [Anonymous, 2017].

2.5 CLINICAL MANIFESTATIONS

On clinical examination the cows were found depressed, febrile, lame (on one limb) with acute swelling of gluteal muscles, anorectic, with complete rumen stasis, high fever (>104°F),

pulse rate of above 110/min. Palpation of the swollen area emitted crackling/crepitating sounds. All the animals were clinically affected within a period of two days indicating the sudden onset and loss.

2.6 PATHOGENESIS

The portal of entry of this organism in cattle is through ingestion of bacterial spores originating from contaminated feed or soil. The ingested spores enter the circulation, upon essential anaerobic conditions (on muscle injury) spores germinate and multiply. The bacteria produce toxins, which in turn actively circulates in blood and causes damage such as severe necrotizing myositis locally in the skeletal muscle and systemic toxemia [Hatheway,1990; Chandler and Gulasekharam, 1974]. The organism invariably causes lesions other than the skeletal muscles at the base of tongue, heart muscle, diaphragm, psoas muscles, on brisket and udder. Characteristic edematous and crepitant swellings develop in the hip, shoulder, chest, back, neck, or elsewhere. At first, the swelling is small, hot, and painful. As the disease rapidly progresses, the swelling enlarges, there is crepitation on palpation. Death occurs within 12–48 hrs. In exogenous infections, spores are introduced into wounds where they germinate in the anaerobic necrotic material, followed by toxin production and release [Quinn *et al*., 1994].

2.7 DIAGNOSIS

The first diagnostic point involves the history by the owner- history of sudden disease and death of the animal and lameness in limbs of the animal. Secondly by the clinical signs- crepitant swellings of the heavy muscles and lameness. The affected muscles are dark red to black, dry and spongy, have a sweetish odour, and are infiltrated with small bubbles but little edema. At times, both *C. septicum* and *C. chauvoei* may be isolated from blackleg lesions, particularly when the carcass is examined $\geq$24 hr after death, which allows time for postmortem invasion of the tissues by *C. sordellii*. Laboratory diagnosis include demonstration of *C. chauvoei* in affected muscle. For confirmatory diagnosis by laboratory examination, samples should be collected from affected muscles, as soon after the death of the animal. The fluorescent antibody test for *C. chauvoei* is rapid and reliable. A PCR is available and reported to be very

good for clinical samples, but not for environmental samples. Conventional isolation and identification of the organism on the basis of morphology, cultural characteristics, biochemical properties, pathogenicity tests, serological and molecular methods are practiced. On post mortem examination, findings in cattle include dark, discoloured, swollen and rancid muscle upon incision of the affected area. A characteristic rancid butter odour emanates from the incised muscle. Upon Gram staining of the culture, the organism will appear as Gram positive rods when examined immediately following death of the animal, but after several hours, samples from the lesions on culturing and Gram staining will show more spores and pleomorphic forms. It is always necessary to differentiate between *C. chauvoei* and *C. septicum* as both are occasionally isolated from the dead animals [Songer, 2010].

2.8 ANTIGENICITY

Immunity against *C. chauvoei* is associated with somatic and flagellar antigens (endogenous antigens) [Tamura and Tanaka,1984; Chandler,1975]. The exogenous antigens for *Clostridium chauvoei* may include: metabolic products and cultural filterates of bacteria which are responsible for pathogenesis of bacteria. The immunoreactive antigens of *C. chauvoei* using 2D gel electrophoresis of the cell wall associated proteins were identified [Usharani *et al.*, 2015].

2.9 VIRULENCE FACTORS

It is postulated that *C. chauvoei* produces some virulence factors or exotoxins, which are the main factors responsible for causing pathogenesis. The major toxins produced by the bacteria include: α toxin (oxygen stable haemolysin), ß toxin (DNase), γ toxin (hyaluronidase) and δ toxin (oxygen labile haemolysin). Moussa (1958) gave a clear picture of the various type of toxins, viz. alpha, beta, gamma and delta in the supernatant of *Clostridium septicum* and *C. chauvoei*. Haemolytic activities of *C. chauvoei* were reported by Ramachandran (1969). Bernheimer's (1944) reported that the oxygen-stable haemolysin was identical with the lethal toxin. In contrast to the late appearance of the oxygen-stable haemolysin, the oxygen-labile haemolysin together with the hyaluronidases and deoxyribonucleases have been shown to appear early, reaching their maximum within the first 18 hr of incubation. Recently it has been

shown that *C. chauvoei* produces a pore-forming cytotoxin and haemolysin, *C. chauvoei* cytotoxin A (*cctA*). [Frey *et al*., 2012] .

2.9.1 ALPHA TOXIN

Information on the alpha-toxins of the organisms is somewhat vague and confusing. Bernheimer (1944) established that the alpha-toxin of *C. septicum* is responsible for the lethal and necrotizing activities of the culture filtrates and also for at least some of the haemolytic activity. Verpoort *et al*. (1966) reported that *C. chauvoei* alpha-toxin has a molecular mass of 27 kDa, but is formed as part of a larger 53.5-kDa complex, which is referred to as the "soluble immunizing component". *C. chauvoei* does not produce titers of alpha-toxin as high as *C. septicum*, and with cultures of the former it is sometimes difficult to detect. Al-Khatib (1969) found that antiserum to *C. septicum* neutralized the lethal activity of both organisms, but antiserum to *C. chauvoei* neutralized the lethality of the homologous organism only. He postulated that *C. septicum* produces two alpha-toxins, alpha-1 and alpha-2, while *C. chauvoei* produces only alpha-1. Moussa (1958) also found that *C. chauvoei* antisera failed to neutralize *C. septicum* alpha-toxin.

2.9.2 BETA TOXIN

The beta toxin of *C. chauvoei* has deoxyribonuclease (DNase) activity and is considered as one of its main virulence factors. These are enzymes having the capacity to degrade highly polymerized deoxyribonucleic acid. The enzyme activity can be made evident on a DNA substrate observing the macroscopic degradation. This enzyme is a heat-stable protein, responsible for nuclear breakdown of muscle cells and is involved in the process of gangrenous myositis triggered by this organism in affected individuals [Cortinas *et al*.,1999; Glenn, 1998]. DNase production is indicative of virulence mechanism in most bacteria including *Clostridium* [Koneman *et al.*, 1997]. DNase detection can be performed *in vitro* by culturing the microorganism on a medium containing DNA as the substrate and macroscopically by checking the degradation. Each strain, from a 24-hour culture is to be seeded on solid medium (Columbia agar containing 5% defibrinated sheep blood) for detection of DNase. Production of the enzyme is revealed on plates by flooding the surface with 1N

HCl. HCl precipitates the DNA, transforming the culture medium translucent [Jeffries *et al.*, 1957]. The appearance in the culture medium of a clear and transparent area around and under the microbial development, indicative of enzymatic digestion, considered as a positive reaction. Areas where no enzymatic activity detected remained opaque after addition of HCl, showing no DNase production . The detection of DNase is significant for an etiological diagnosis and to determine the virulence of the bacterial strain isolated in the laboratory. Princewill and Oakley (1976) found that the beta toxin is heat stable, activated or inhibited by metal ions and chelating agents, and having perceptibility with ammonium sulphate. A molecular mass of 45 kDa for the enzyme has been estimated.

2.9.3 GAMMA TOXIN

Gamma toxin of *Clostridium chauvoei* is a hyaluronidase, an enzyme that hydrolyzes glycosidic bonds between N-acetylglucosamine and glucuronic acid residues of hyaluronic acid. This enzyme is readily inactivated by heat treatment. Princewill and Oakley (1976) found that the gamma-toxin of *C. septicum* was much more sensitive to heat than that of *C. chauvoei*. The genome of *Clostridium chauvoei* reveals two different hyaluronidase genes [Frey and Falquet, 2014]. Bacterial hyaluronidases, enzymes capable of breaking down hyaluronate, are produced by a number of pathogenic Gram-positive bacteria that initiate infections at the skin or mucosal surfaces. Hyaluronidase are the enzymes that are able to breakdown the substrate hyaluronate (hyaluronic acid , hyaluronan); however some of these enzymes are also able to cleave chondroitin sulphate. Since hyaluronate is a major constituent of the ground substance of most connective tissues, particularly the skin, hyaluronidase may be an essential component in enabling the spread of the pathogens from the initial site of infection [Hynes and Walton, 2000]. The role played by this toxin in the pathogenesis of blackleg is not clear. Gram-positive organisms capable of producing hyaluronidase include various species of *Streptococcus, Staphylococcus, Peptostreptococcus, Propionibacterium, Streptomyces* and *Clostridium.*

2.9.4 DELTA TOXIN

The delta-toxin produced by this organism is an oxygen-labile haemolysin similar to counterparts produced by other Clostridia (e.g., the theta-toxin of *C. perfringens* and

tetanolysin of *C. tetani*). It is neutralized by antistreptolysin-O serum. The delta-toxins of *C. chauvoei* and *C. septicum* are cross-neutralized by their opposite antisera, but the neutralizing potency of the homologous antisera is clearly stronger. The haemolytic potency of the toxins is inactivated by oxidizing agents such as hydrogen peroxide and iodine; the inactivation can be reversed by treatment with reducing agents such as sodium thioglycollate and hydrogen sulfide. Haemolysis produced by the delta toxin occurs more rapidly than that produced by the alpha toxin [Hatheway, 1990].

2.9.5 SIALIDASE (designated as NanA)

Sialidase is encoded as a precursor protein of 722 amino acids with a 26 amino acid signal peptide. The molecular weight of *C. chauvoei* sialidase (neuraminidase) is 65 kDa and it is reported that native enzyme exist as a dimeric protein of 135 kDa [Useh *et al.*, 2006]. The mature sialidase has a calculated molecular mass of 81 kDa and contains the carbohydrate binding module 32 (CBM32, or F5/8 type C domain), the sialic acid binding module CBM40 and the enzymatically active sialidase domain found in all pro- and eukaryotic sialidases. Sialidase activity does not require the CBM32 domain. The nanA protein is secreted by *C. chauvoei* as a dimer [Vilei *et al*., 2011]. The NanA gene was found to be conserved and sialidase activity was found in *C. chauvoei* strains isolated over a period of 50 years from various geographical locations. Antiserum directed against a recombinant 40 kDa peptide containing CBM40 and part of the enzymatically active domain of *nanA* neutralized the secreted sialidase activity of all *C. chauvoei* strains tested. Sialidases, or neuraminidases are enzymes that cleave N-acetylneuraminic acid from carbohydrate polymers, such as mucin, glycoproteins, gangliosides and other sialoglycoconjugates, located on many mammalian cell membranes. Sialidase may have an important role in blackleg by degrading tight junctions upon cleavage of sialic acids at a high rate, thus allowing the bacterium to spread through host tissue [Hang'ombe *et al*., 2006]. Cleavage of sialic acid results in decreased rigidity of the cell surface, thereby facilitating cell motility and thus rendering the target site vulnerable to massive attack by the pathogen that rapidly spreads in the infected tissue. A sialidase activity has been reported in *C. chauvoei* [Useh *et al.*, 2006], but the sialidase enzyme has not yet been characterized in detail [Nazir *et al.*, 2012; Sultana *et al*., 2008]. Furthermore, the molecular and genetic basis of

sialidase in *C. chauvoei* is still unclear. *C. chauvoei* sialidase is found as a 150 kDa protein, in the form of a dimer of two NanA peptides with an apparent molecular mass of 72 kDa. The full length sialidase gene *nanA* of *C. chauvoei* strain JF4135 consists of 2319 bp. Recombinant sialidase protein of *C. chauvoei* has been shown to have diagnostic potential [Singh, 2013].

2.9.6 FLAGELLA

Phase variation in motility and flagellation occurs in *C. chauvoei,* and that the flagella are associated with the full expression of virulence. The flagella of C. *chauvoei* are involved in inducing immune resistance mechanisms, as demonstrated by the mouse protection test [Tamura *et al.*, 1984]. Flagella have therefore, been considered as one of the protective antigens contained in the vaccine against blackleg in domestic animals. Flagella of C. *chauvoei* are associated with the expression of full virulence. Further studies on the role of flagella in infection of *C. chauvoei* are necessary in order to elucidate the pathogenicity of this species [Kojima *et al.*, 2000]. The flagella of *C. chauvoei* are of special interest for two reasons: first, they seem to be a protective antigen [Tamura *et al*., 1984]; second, the flagellum of *C. chauvoei* is a virulence factor [Tamura *et al*., 1995]. The coding region of the *C. chauvoei* flagellin gene is *fliC*. SDS-PAGE profile of purified flagella showed that a major protein band with a molecular mass of 46 kDa, corresponding to the flagellin monomer, and at least two minor protein bands with molecular masses of approximately 73 and 100 kDa were found. The amino acid composition of *C. chauvoei* flagellin was similar to the flagellin of *Salmonella typhimurium* and *Bacillus subtilis*. In addition, *C. chauvoei* flagellin monomer shared limited sequence homology with the N-terminal amino acid sequence reported for other bacterial flagellins. N-terminal sequences of two minor bands corresponded to the flagellin monomer, indicating that higher molecular mass bands were polymeric forms of the flagellin monomer [Kojima *et al*., 1999]. A recombinant flagellin based ELISA for detection of *C. chauvoei* has been developed [Usharani *et al.*, 2015].

2.10 WHOLE GENOME SEQUENCE ANALYSIS OF *C. chauvoei*

Recently, whole genome of *C. chauvoei* has been sequenced [Frey and Falquet, 2014] and the complete genome is available at NCBI website (NCBI Accession No.

NZ_LT99839.1). Frey and Falquet (2014) has classified the primary virulence factors of *C. chauvoei* (strain JF4335) under two different heads: Haemolysins and Non-haemolysins. Haemolysins include Panton Valentine leucocidin CctA, Haemolysin III, Haemolysin XhlA, Haemolysin A and Non-haemolysins include Sialidase, Hyaluronidase, Collagen binding protein, Internalin A.

2.10.1 PANTON VALENTINE LEUCOCIDIN CctA:

It is a 32kDa β-barrel pore-forming toxin belonging to the leucocidin superfamily of bacterial toxins. It has been analysed in detail both genetically and biochemically [Cortinas *et al.*, 1999]. This leucocidin CctA represents the main haemolytic and cytotoxic activity of *C. chauvoei*. Furthermore, guinea pigs vaccinated with inactivated recombinant CctA were protected against challenge with virulent *C. chauvoei*, thus showing its central role in virulence and its potential in immune protection. The gene *cctA* is well conserved in *C. chauvoei* strains isolated over a period of 50 years from different parts of the world. The protein was highly cytotoxic to embryonic calf nasal epithelial cells and had high hemolytic activity against sheep erythrocytes. *cctA*, a novel secreted toxin of *C. chauvoei* that is responsible for the major cytotoxic activity of *C. chauvoei* as determined on bovine ECaNEp cells [Frey *et al.*, 2012]. This toxin also confers on *C. chauvoei* its characteristic strong haemolytic activity. While other toxins have been postulated as the major toxins of *C. chauvoei* , monospecific anti-CctA antibodies fully neutralized all cytotoxic and haemolytic activity found in *C. chauvoei* culture supernatants showing that CctA represents the major cytotoxin and haemolysin of *C. chauvoei* [Frey *et al.*, 2012]. Most importantly, animals vaccinated with recombinant CctA, were highly protected against challenge with virulent *C. chauvoei* [Frey *et al.*, 2012]. It has also been shown that recombinant CctA protein could also be used for diagnosis of *C. chauvoei* infections [Sophia, 2013].

2.10.2 HAEMOLYSIN A

Haemolysin A belongs to the FtsJ superfamily of RNA methyltransferases. Its role in pathogenesis is questionable. The ftsJ protein is a well conserved heat shock protein that is found not only in prokaryotes, but also in archaea and eukaryotes, where it is responsible for

methylation of the 23S rRNA supposedly in the late maturation phase of the ribosome. Hence *ftsJ* could represent a house keeping gene [Frey and Falquet, 2014].

2.10.3 HAEMOLYSIN XhlA

It is small protein of 8.8kDa. Genes encoding homologues to XhlA are found in many Gram-positive bacteria, in particular in insect pathogen species of *Paenibacillus, Bacillus, Clostridium*, but also in some *Clostridium botulinum* and in *Clostridium glycolyticum* strains. Haemolysin XhlA was primarily shown as a major virulence factor of *Xenorhabdus nematophila* which is a pathogen for a variety of insects [Cowles and Goodrich-Blair, 2005]. It is therefore questionable if XhlA plays a role in pathogenicity of blackleg by *C. chauvoei*, or if the gene is a requisite for evolution of *Clostridium* species [Frey and Falquet, 2014].

2.10.4 HAEMOLYSIN III

Though the major haemolytic protein is CctA, haemolysin III may also play a significant role in the pathogenesis of *Clostridium chauvoei*. The putative haemolysin III represents a protein of 220 amino acids with a molecular mass of 25 kDa and a calculated pI of 9.1 and characteristic domains of the haemolysin III-superfamily. This haemolysin could represent the 27 kDa haemolysin that was identified in *C. chauvoei* strain C6H by Hang'ombe and co-workers [Hang'ombe *et al*., 2000]. A gene encoding a highly similar Haemolysin III with 58% identity and 75% similar amino acid to that in *C. chauvoei* is found in nearly all *C. perfringens* strains that were analysed genetically in detail. Haemolysin III is referred as haemolysin D in *C. perfringens*. Putative haemolysins of the haemolysin III-superfamily are very widely spread in Gram-positive bacteria and are found in pathogenic as well as in commensal and environmental species such as *Flavibacterium sp., Bacillus sp., Stenotrophomonas sp., Paenibacillus sp., Photobacterium sp., Desulfitibacter sp., Desulfobacterium sp.* where it is named as haemolysin III or alternatively haemolysin D [Frey and Falquet, 2014]. It has been described as one of the haemolysin-toxins of *Bacillus cereus*. The role of haemolysin III in pathogenicity of *C. chauvoei* is still unclear.

In case of *Bacillus cereus*, haemolysis by haemolysin III occurs in atleast three steps:

(i) The temperature dependent binding of the HlyIII monomers to the erythrocyte membrane

(ii) The temperature dependent formation of transmembrane pore by multiple molecules of the haemolysin

(iii) Temperature independent erythrocyte lysis [Baida and Kuzmin, 1996].

It has been reported in *Bacillus cereus* that Haemolysin III induces haemolysis by multi-hit mechanism indicating that haemolysin binds to the erythrocyte membrane in monomeric form and multiple monomers are required to lyse one cell.

Haemolysin III gene has also been characterized in *Vibrio vulnificus*, a causative agent of both serious wound infections and fatal septicemia. A gene (*hlyIII*) encoding a haemolysin was cloned and sequenced from *V. vulnificus*. Nucleotide sequence analysis predicted an open reading frame of 642 bp encoding a 214 amino acid polypeptide that showed 48% sequence identity to the haemolysin III of *Bacillus cereus*. When HlyIII of *V. vulnificus* was expressed in *Escherichia coli*, crude extracts exhibited haemolytic activity similar to that of haemolysin III from *Bacillus cereus*. A *hlyIII* isogenic mutant was constructed via insertional inactivation and showed an attenuated virulence compared with the wild-type strain when this mutant was administered intraperitoneally in mice [Chen *et al*., 2004].

2.11 HAEMOLYSIS PRODUCED BY *Clostridium chauvoei*

Cow erythrocytes are most susceptible to the haemolysis caused by *C. chauvoei* followed by sheep and then chicken while horse erythrocytes are resistant. The reason why haemolytic activity against erythrocytes from various animal species differs may be due to the existence of toxin binding receptors on the surface of erythrocytes. Erythrocytes that do not have these receptors will not to be sensitive to the toxin. Studies have shown that haemolysin-producing bacteria are more virulent to animals than haemolysin lacking bacteria [Cavalieri and Snyder, 1982; Smith, 1975].

2.12 TatD HYDROLASE

TatD is shown to be a cytoplasmic protein, which exhibits magnesium-dependent DNase activity. TatD is a soluble protein [Wexler *et al*, 2000] and is unlikely to be a permanent component of the Tat translocon. At present, no information is available on its mode of action; the Δ*tatD* strain has no clear phenotype, but the studies on the purified protein showed that it has DNase activity [Wexler *et al.*, 2000].

In *E.coli*, TatD was encoded by a *Tat* operon that encodes Tat proteins, TatA, TatB, TatC and TatD, for protein transport via the Tat (Twin-Arginine Translocation) pathway [Sargent *et al.*,1998] .

For detection of the DNase activity of the TatD Hydrolase, Methyl Green DNA agar test and Toluidine Blue DNA agar test can be used. The DNase produced by the organism causes the DNA to depolymerize and all these tests of DNase depends on the demonstration of the depolymerization of its substrate, DNA, by the enzyme. In the methyl green DNA agar test, the positive reaction is observed as the presence of colourless zones around the bacterial colonies or the protein lysate against the green background. While in Toluidine blue DNA agar test the positive reaction is observed as the presence of pink zone around the colonies or the protein lysate against the blue background [Chaudhari and Singh, 1992].

2.13 RECOMBINANT PROTEIN PRODUCTION IN PROKARYOTIC EXPRESSION SYSTEM

Use of prokaryotic expression systems offers an economical method to achieve production of large amounts of recombinant protein. The facility provides technical support for production of recombinant protein in bacteria. Bacterial expression systems for heterologous protein production are attractive because of their ability to grow rapidly and at high density on inexpensive substrates, their often well-characterized genetics and the availability of an increasingly large number of cloning vectors and mutant host strains. To produce high levels of protein, it is often useful to clone the gene downstream of a well-characterized, regulated promoter. The gram-negative bacterium *E. coli* is the most commonly used organism for heterologous protein production. One of the reasons seems to be that this

organism is very well-known and established in each laboratory. Therefore the *E. coli* systems are also most commonly used for industrial and pharmaceutical protein production. Large-scale production systems are established. As *E.coli* lacks post translation machinery, absence of post translational modifications of the recombinant protein, and formation of inactive protein due to inclusion body formation are considered as its drawbacks [Sahdev *et al*., 2008]. A disadvantage for therapeutic use of produced recombinant proteins in *E. coli* is the accumulation of lipopolysaccharide (LPS), generally referred as endotoxins, which are pyrogenic in humans and other mammals. Proteins for this application must be purified in a second step to become endotoxin-free [Petsch and Anspach, 2000].

DNA encoding a target protein is cloned downstream of a promoter in an expression vector. This vector is then introduced into a host cell, and the cell's protein synthesis machinery produces the desired protein. Protein expression is a subcomponent of gene expression. It consists of the stages after DNA has been transcribed to messenger RNA (mRNA). The mRNA is then translated into polypeptide chains, which are ultimately folded into proteins. Protein expression is commonly used to denote the measurement of the presence and abundance of one or more proteins in a particular cell or tissue.

Expression systems are genetic constructs (a gene encoded by DNA) that are designed to produce a protein, or an RNA (ribonucleic acid), either inside or outside a cell. Expression systems are used in research and in the commercial production of enzymes or therapeutics. Protein expression refers to the way in which proteins are synthesized, modified and regulated in living organisms. Proteins are synthesized and regulated depending upon the functional need in the cell. The blueprints for proteins are stored in DNA and decoded by highly regulated transcriptional processes to produce messenger RNA (mRNA). The message coded by an mRNA is then translated into a protein. Transcription is the transfer of information from DNA to mRNA, and translation is the synthesis of protein based on a sequence specified by mRNA. In prokaryotes, the process of transcription and translation occur simultaneously. The translation of mRNA starts even before a mature mRNA transcript is fully synthesized. This simultaneous transcription and translation of a gene is termed coupled transcription and translation.

Recently, Expresso Rhamnose SUMO cloning and expression system has been shown to efficiently express the heterologous genes in *E. cloni* cells. The Expresso T7 SUMO Cloning and Expression System is a simple method for rapid cloning and expression of proteins in *E. cloni*. The system uses an engineered form of the SUMO protein (Small Ubiquitin-like MOdifier) as a fusion partner to aid the expression and purification of difficult target proteins. In Expresso Rhamnose SUMO Cloning and Expression System, a PCR product containing the gene of interest is cloned as a fusion to the solubility-enhancing, cleavable SUMO tag under the control of the L-rhamnose-inducible rhaP BAD promoter harbored on the pRham™ N-His SUMO Vector. Because this promoter is recognized by the bacterial RNA polymerase, a single host strain is used for both clone construction and protein expression [Anonymous, 2016]. This single-host strategy allows a streamlined workflow compared to systems requiring separate hosts for cloning and expression [Giacalone, *et al.*, 2006].

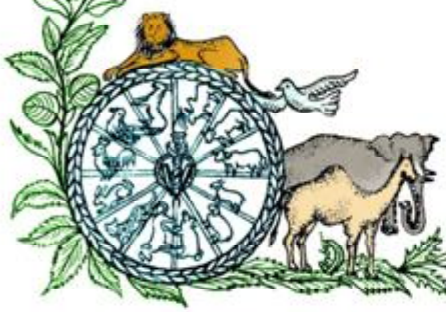
The pETite vectors provide an improved alternative to the most common pET expression vectors. The small size of the pETite vectors (2.2-2.5 kb) facilitates cloning of large inserts and performing DNA manipulations, such as site-directed mutagenesis. They have transcriptional terminators to prevent unwanted transcription into or out of the cloned sequence. The pETite vectors do not harbour a gene for the LacI repressor protein. Instead, abundant LacI repressor is provided by the HI-Control™ BL21(DE3) cells. The pETite N-His SUMO Kan Vector is pre-linearized for instant, directional cloning of inserts. The vector includes signals for expression, including the T7-lac promoter, ribosome binding site and translational start and stop codons. The vector is designed for expression of the target protein as a fusion with an amino-terminal 6xHis-SUMO tag, which has been shown to increase the yield and enhance the solubility of a variety of proteins [Anonymous, 2016].

The pET System is the most powerful system yet developed for the cloning and expression of recombinant proteins in *E. coli*. Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is so selective and active that almost all of the cell's resources are converted to target gene expression; the desired product can comprise more than 50% of the total cell protein a few hours after induction. Target genes are initially cloned using hosts that do not contain the T7 RNA polymerase

gene, thus eliminating plasmid instability due to the production of proteins potentially toxic to the host cell.

In pQE30 vectors, high level protein expression is based on the T5 powerful promoter, which has been optimized with an operator element consisting of two *lac* operator sequences that increase *lac* repressor binding and ensure tight repression of the T5 promoter. The 6X His-tag affinity tag can be removed by using pQE30-Xa plasmid which contains the FXa proteolytic recognition site downstream from the 6X His-tag.

Materials and Methods



3 MATERIALS AND METHODS

3.1. MATERIAL

3.1.1. Bacteria

The *Clostridium chauvoei* ATCC 10092, *E. cloni* 10G cells (Lucigen Corporation, Wisconsin, USA), BL-21 cells (Lucigen Corporation, Wisconsin, USA), M-15 cells were obtained from Anaerobe Laboratory, Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar.

3.1.2. Vectors

pRham-N-His-SUMO-Kan Vector (Lucigen Corporation, Wisconsin, USA), peTite SUMO vector (Lucigen Corporation, Wisconsin, USA), pQE30Xa vector (Qiagen, USA), pET32a vector (Invitrogen) were used for the prokaryotic expression.

3.1.3. Oligonucleotide primers

The primer set based on 16S-23S rDNA spacer region of *C. chauvoei* (Sasaki *et al.*, 2001) was used to characterize the *C. chauvoei* culture.

Forward primer: 5'-GAAATTGCACATGAATTAAA-3'

Reverse primers: 5'-GGATCAGAACTCTAACCTTTCT-3'

Primer set based on *cctA* gene of *Clostridium chauvoei* were obtained from Anaerobe lab, IVRI:

Forward primer: 5'- CGCGAACAGATTGGAGGTATAAAAAGAATATTAATGCTT-3'

Reverse primer: 5'- GTGGCGGCCGCTCTATTAATCATTAAAACGATTATATTC-3'

3.1.4. Chemicals and other molecular biology reagents

Acrylamide, bis-acrylamide, tween-20, potassium chloride, potassium acetate, magnesium sulphate, Dextrose and Tris base were procured from Sigma Chemical co., USA.

TEMED, Ammonium per sulfate, Coomassie brilliant blue R-250, Urea, Glacial acetic acid, Tryptone, Glycerol were from SRL, India. L-rhamnose (Bio Basic Inc, Canada) and Luria Bertini agar, Skimmed milk (Difco), SOC medium (Invitrogen), Taq polymerase, dNTPs, DNA ladder, DNase I, Gel loading dye, Ethidium bromide, Tango buffer, Red Buffer, *Hind III*, *Sac I*, *Nco I* enzymes were purchased from MBI Fermentas, USA.

Sodium dihydrogen phosphate, disodium hydrogen phosphate, potassium dihydrogen phosphate were from SRL, India. EDTA, Tris HCl, Agarose, Kanamycin, Ampicillin, Tris, Urea, Glycine, L-Arginine, IPTG, PEG-8000, NaOH, $MgCl_2$, Fish sperm DNA, Toluidine Blue-O dye were from Amresco. Methanol, Sodium dodecyl sulphate were obtained from Merck, India. Yeast extract, NaCl, Bradford reagent and Dialysis membrane from Hi-media, India; Ni-NTA agarose (Qiagen), nitrocellulose membranes, hydrochloric acid (SD Fine Chemicals, India).

3.1.5. Media, Buffers and Reagents

The details of the media, buffers and the other reagents used in this study are given in the appendix.

3.1.6. Plasmid Isolation kit from Qiagen, Germany. Genomic DNA isolation kit and Gel extraction kit were from ThermoScientific, USA.

3.1.7. Glass ware and plastic ware

The plastic ware, used in the present study, were purchased from Tarsons (India). The glassware were from Borosil (India) and Schott Duran (Germany).

3.1.8. Equipments

Gel documentation system (Alpha Innotech Corp.,USA)

Ice Flaker Machine (Harrison Scientific instruments, India)

Orbital shaker incubator (Nu Brunswick,Edision, USA)

Refrigerated micro centrifuge (ThermoFisher Scientific , USA)

Water bath (JSGW, Ambala, India)

Thermocycler (Agilent Technologies, USA)

Variable Volume micropipettes (Eppendorf, Germany)

Sorval RC 5C plus high speed refrigerated centrifuge (DuPont, USA)

Electronic balance (Mettler AT261 and Afcoset, India)

-20 °C Deep freezer (Blue star)

-80 °C Biomedical freezer (New Brunswick, USA)

Horizontal gel electrophoresis apparatus (Bangalore Genei, India)

Vertical gel electrophoresis apparatus (Bio-Rad, USA)

Nanovue plus (General electronics)

3.2. Methods

3.2.1. Revival of glycerol stock of *C. chauvoei*

C. chauvoei ATCC 10092 culture was revived in ATCC 2107 media (Reinforced Clostridial medium). Glycerol stock of *C. chauvoei* culture 100 µl was inoculated in 10 ml ATCC 2107 media in the serum vial and incubated at 37 °C for 48 hrs. After 48 hrs, culture was taken out and stained by Gram staining procedure which revealed Gram positive bacilli in short chains of 2-3 bacilli.

3.2.2. Characterization of *C. chauvoei*

3.2.2.1. Gram staining

The culture smears were stained by Gram's staining as described by Quinn *et al*. (1994). The slides were air dried and viewed under oil immersion microscope.

3.2.2.2. Molecular identification of *C. chauvoei*

a) Isolation of genomic DNA from *C. chauvoei*

(i) Snap chill method of isolation of genomic DNA

The growth of *C. chauvoei* cells in late logarithmic phase culture were pelleted by centrifugation, washed with sterile PBS, boiled for 10 mins and immediately cooled on ice to release the genomic DNA. The genomic DNA in the supernatant was separated from the cell debris by brief spinning. This was used as the template for the PCR amplification.

(ii) Genomic DNA isolation by kit method (ThermoScientific, USA)

2 ml *Clostridium chauvoei* culture taken in a microcentrifuge tube was centrifuged for 10 min at 5000*g and supernatant was discarded. Pellet was resuspended in 180 µl of the bacterial lysis buffer (20mM Tris-HCl, pH=8.0, 2mM EDTA, 1.2% Triton X-100, lysozyme-20mg/ml) added immediately before use. It was incubated for 30 min at 37 °C. 200 µl of Lysis solution and 20µl of Proteinase K was added. It was mixed thoroughly by vortexing to obtain a uniform suspension and then the sample was incubated at 56°C, vortexing occasionally until the cells are completely lysed. 20µl of RNase A solution was added, mixed by vortexing and incubated for 10 min at room temperature. 400 µl of 50% ethanol was added and mixed by pipetting or vortexing. The prepared lysate was added to the Genomic DNA purification column inserted in a collection tube. The column was centrifuged for 1 min at 6000*g. Collection tube containing the flow-through solution was discarded. The purification column was placed into a new 2 ml collection tube. 500µl of Wash buffer I was added (with ethanol added)and centrifuged for 1 min at 8000*g. The flow-through was discarded and the purification column was placed back into the collection tube. 500µl of Wash buffer II (with ethanol added) was added to the column. Centrifuged for 3 min at 12000 rpm. The collection tube containing the flow-through solution was discarded and the column was transferred into a sterile 1.5 ml microcentrifuge tube. 100 µl of Elution buffer was added to the centre of the Genomic DNA purification column to elute the genomic DNA. It was then incubated for 2 min at room temperature and centrifuged for 1 min at 8000*g. The column was discarded and genomic DNA can be stored at -20°C.

3.2.2.3. PCR for 16S-23S rDNA spacer region and *cctA* gene for confirmation of *C. chauvoei*

PCR amplification of 16S-23S rDNA was performed as described by Sasaki *et al*. (2001). The identification of *C. chauvoei* was carried out using the specific primers (IGSCS and 23UPCH primers) targeting 16S-23S rDNA spacer region and *cctA* gene. Genomic DNA from *C. chauvoei* was used as template for PCR amplification.

Table 1: 16-23s rDNA spacer and cctA gene based PCR reaction mixture

Reaction mixture	Volume (µl)
10X Taq buffer	2.5
dNTPs (10 mM)	0.5
Primers (10 pmoles/µl)	
Forward primer	0.5
Reverse primer	0.5
25 mM $MgCl_2$	0.5
Taq DNA Polymerase (5 U/ µl)	0.25
Nuclease free water upto	25

About 1.5 µl of genomic DNA was added and then the following cycling conditions were standardized for the amplification of the gene of interest.

Step 1: Initial Denaturation	94 °C for 5 min
Step 2: Denaturation	94 °C for 1 min
Step 3: Annealing	54 °C for 1 min (for 16S-23S rDNA spacer) and 58 °C (*cctA*) for 1min
Step 4: Extension	72 °C for 1min
Steps from denaturation to extension were repeated 34 times	
Step 5: Final extension	72 °C for 10 min

The actual PCR was carried out using the same reaction mixture mentioned above in a 25 µl reaction. The amplified product was visualized by electrophoresis on 1.3% agarose gel.

3.2.3. Designing of primers for amplification of *tatD and hlyIII* gene of *C. chauvoei*

The primers were designed from available sequence of *tatD* gene to aid in cloning into pRham-N- His SUMO-Kan Vector *and hlyIII* to aid in cloning into pRham-N- His SUMO-Kan Vector, peTite SUMO vector, pQE30Xa vector and pET32a vector

SUMO-TatD-For- 5'-CGCGAACAGATTCGAGGTGAAGGAAAATATTTAATTTTT-3'

SUMO-TatD-Rev- 5'-GTGGCGGCCGCTCTATTATATTCTATTTTCAAGCAAGTC-3'

SUMO-Hly-III-For-5'-CGCGAACAGATTGGAGGTTCAAAGAGAGATTAATAAG-3'

SUMO-Hly-III-For2- 5'-CGCGAACAGATTGGAGGTGAGATTAATAAGAGTGTAGAA-3'

SUMO -Hly-III-Rev-5'-GTGGCGGCCGCTCTATTATAGTAATACACATAAAAATAC-3'

pET32a -Hly-III-For-5'-GCGCCCATGGCAAAGAGAGAGATTAATAAG-3'

pET32a -Hly-III-Rev-5'-GCGCAAGCTTTTATAGTAATACACATAAAAATAC-3'

pQE30Xa-Hly-III-For-5'-ATCCATGGGCAAATGCAATAACTCATGGA-3'

pQE30Xa -Hly-III-Rev-5'-GCAAGCTTTTAATGCAATACACTACCTGCCAT-3'

3.2.4. PCR Amplification of *tatD* and *hlyIII* genes of *C. chauvoei*

A gradient PCR was carried out initially to optimize the annealing temperature, with different temperatures (48-57 °C) for SUMO primers and at temperatures (50-65°C) in case of primers of other vectors in a gradient thermal cycler (Eppendorf, Mastercycler). After this the gene was amplified in a suitable annealing temperature using *pfu*:Taq polymerase (1:15). The reaction was carried out in a standard 25 µl reaction volume using the following reagents in a PCR tube.

Table 2: *tatD* and *hlyIII* gene amplification based PCR reaction mixture

Reaction mixture	Volume (µl)
10X Taq buffer	2.5
dNTPs (10 mM)	0.5
Primers (10 pmoles/µl)	
Forward	0.5
Reverse	0.5
25 mM $MgSO_4$	0.5
Pfu:Taq Polymerase (1:15)	0.25
Nuclease free water upto	25

About 1.5 µl of genomic DNA was added and then the following cycling conditions were standardized for the amplification of the gene of interest.

Step 1: Initial Denaturation	94 °C for 5 min
Step 2: Denaturation	94 °C for 1 min
Step 3: Annealing	51 °C for 1min (*tatD*)
	51 °C for 1 min (*hlyIII* in pRham-N-His SUMO Kan vector and pETite-N-His SUMO Kan Vector)
	52 °C for 1 min (*hlyIII* in pET32a)
	55 °C for 1 min (*hlyIII* in pQE30Xa)
Step 4: Extension	72 °C for 1min
Steps from Denaturation to Extension were repeated 34 cycles	
Step 5: Final extension 72 °C for 10 min	

The actual PCR was carried out using the same reaction mixture mentioned above in a 25 ml reaction. The amplified product was visualized by electrophoresis on 1.3% agarose gel.

3.2.5. Agarose gel electrophoresis

Agarose gel (1.3%) was prepared by boiling the agarose in an appropriate volume of 1X TAE (Appendix). It was then allowed to cool to about 50 °C and ethidium bromide (10 µg/ ml) was added to a final concentration of 0.5 µg/ml. About 40 ml of agarose gel was poured into the casting tray and was allowed to solidify at room temperature for 20 mins following insertion of the comb. Prior to electrophoresis, 2 µl of 6X loading dye (MBI, Fermentas) was added to 5 µl of PCR product and the samples were loaded in each well of the gel along with a 100 bp DNA ladder (Fermentas) in a designated well. After the electrophoresis, the gel was examined in a gel documentation system (Alpha Innotech Corp., USA).

3.2.6. Gel extraction of PCR product

The gel extraction of DNA fragments was carried out using Mini Elute Gel extraction kit (ThermoScientific, USA) following the manufacturer's instructions. The gel containing the

DNA fragment was excised using a clean scalpel and placed into a pre-weighed 1.5 ml tube and weighed. The weight of the gel slice was recorded. Then 1:1 volume of Binding buffer was added to the gel slice (100 µl of binding buffer for every 100 mg of agarose gel). The gel mixture was incubated at 50-60 C for 10 min or until the gel slice is completely dissolved. The tube contents were mixed thoroughly and upto 800 µl of the solubilised gel solution was transferred to the GeneJet purification column. It was then centrifuged for 1 min. Flow-through was discarded and the column was placed back into the same collection tube. 700 µl of Wash buffer was added to the column and centrifuged for 1 min. Flow-through was discarded and the column was placed back into the same collection tube. The empty column was centrifuged for additional 1 min to completely remove the residual wash buffer. The column was transferred into a sterile 1.5 ml microcentrifuge tube and 20-50 µl of Elution buffer was added to the centre of the column. Centrifuged for 1 min. The column was discarded and purified DNA was stored at -20^{o}C

3.2.7. Cloning of the *tatD* and *hlyIII* gene

In order to clone the gene into the expression vector, the amplified *tatD* and *hlyIII* genes (PCR products) were resolved and visualized in 1.3% agarose gel containing 0.5 µg/ml ethidium bromide. Finally, the PCR product was purified as described earlier using ThermoScientific gel extraction kit.

3.2.8. Ligation of *tatD* and *hlyIII* gene into pRham-N-His SUMO-Kan expression vector and transformation of *E. cloni* 10G chemically competent cells

Transformation was performed as per manufacturer's protocol (Lucigen, USA). The *E. cloni* 10G competent cells were thawed on the ice for 10 mins and transferred into a pre cooled 15 ml centrifuge tube. The 2 µl (25 ng) of pRham-N-His SUMO-Kan Vector DNA was added with 100 ng of the purified PCR product and mixed gently by stirring within the cells without pipetting. The tube containing the mixture of cells and DNA was incubated on ice for 30 mins, and then heat shocked the cells at 42 °C for 45 sec. The tube was kept on ice for further 2 mins and 960 µl of recovery medium added. The cells were incubated at 37 °C in a shaking incubator for 2 hrs and plated on the LB agar containing Kanamycin (30 µg/ml).

3.2.9. Ligation of *tatD* and *hlyIII* gene into pETite-N-His SUMO Kan expression vector and transformation of *E. cloni* 10G chemically competent cells and then transformation of BL-21 competent cells

Transformation was performed as per manufacturer's protocol (Lucigen, USA). The *E. cloni* 10G competent cells were thawed on the ice for 10 mins and transferred into a pre cooled 15 ml centrifuge tube. The 2 µl (25 ng) of pETite SUMO Vector DNA was added with 100 ng of the purified PCR product and mixed gently by stirring within the cells without pipetting. The tube containing the mixture of cells and DNA was incubated on ice for 30 mins, and heat shocked at 42 °C for 45 sec. The tube was kept on ice for further 2 mins and 960 µl of the recovery medium added. The cells were incubated at 37 °C in a shaking incubator for 2 hrs and plated on the LB agar containing Kanamycin (30 µg/ml). The colonies obtained were then subcultured in LB broth containing Kanamycin (30 µg/ml). Plasmid was extracted from the transformed culture and this plasmid was then transformed into BL-21 competent cells (Lucigen, USA) and plated onto LB agar containing Kanamycin (30 µg/ml).

3.2.10. Ligation of *hlyIII* gene into expression vector- pET32a and transformation of *E.coli* BL-21 cells

For cloning the *hlyIII* gene in pET32a expression vector , both the PCR product and the and the vector were digested separately with *NcoI* and *Hind III* restriction enzymes. After incubation at 37°C for 4 hrs, the PCR product and the vector plasmid DNA were resolved and visualized in 1.2 % agarose gel containing 0.3 µg/ml ethidium bromide. The bands were excised and purified using the ThermoScientific GeneJet gel extraction kit.

3.2.10.1 Ligation of *hlyIII* into pET32a expression vector:

NcoI and *Hind III* digested *hlyIII* and pET32a prokaryotic expression vector were ligated with T4 DNA ligase enzyme as described below:

Table 3:

NcoI and *Hind III* digested vector	6 µl
NcoI and *Hind III* digested PCR product	4 µl
T4 DNA Ligase	1 µl
10X Ligase buffer	2.5 µl
NFW	6.5 µl

The above reaction was incubated overnight at 4°C.

3.2.10.2 Transformation of *E.coli* BL-21 competent cells

For making competent cells : 50 ml overnight grown culture of BL-21 was taken and it was subcultured in LB broth and kept at 37°C in shaker incubator till O.D. reaches 0.25-0.4. 1X TSS (Appendix) was kept on ice. 1 ml of the culture (kept on ice for 5 min) was taken and pelleted and resuspended in 100 µl TSS (kept on ice). Gently mix it and cells can be used or stored at -80°C immediately.

Transformation: TSS treated cells were kept on ice. 100 pgm –10 ng DNA (ligated mixture) was added to the cells. It was then gently mixed and kept on ice for 15-30 min. Heat shock was given for 90 sec at 42°C. Then it was immediately transferred to ice and kept for 10 min. Then 1 ml LB or SOS medium was added to it and kept in shaker incubator at 37°C for 1.5 hrs at 200 rpm. Then it was plated onto LB plate containing 100 µg/ml .

3.2.11. Ligation of *hlyIII* gene into expression vector- pQE30Xa and transformation of *E.coli* M-15 cells

For cloning the *hlyIII* gene in pQE30Xa expression vector , both the PCR product and the vector were digested separately with *Sac I* and *Hind III* restriction enzymes. After incubation at 37°C for 4 hrs, the PCR product and the vector plasmid DNA were resolved and visualized in 1.3 % agarose gel containing 0.3 µg/ml ethidium bromide. The bands were excised and purified using the ThermoScientific GeneJet gel extraction kit.

3.2.11.1 Ligation of *hlyIII* into pQE30Xa expression vector:

Sac I and *Hind III* digested *hlyIII* and pQE30Xa prokaryotic expression vector were ligated with T4 DNA ligase enzyme as described below:

Table 4:

Sac I and *Hind III* digested vector	6 µl
Sac I and *Hind III* digested PCR product	8 µl
T4 DNA Ligase	1 µl
10X Ligase buffer	2.5 µl
PEG 4000	1 µl
NFW	1.5 µl

The above reaction was incubated overnight at 4^0C.

3.2.11.2 Transformation of *E.coli* M15 competent cells

The transformation procedure was performed using Transformaid kit as per the manufacturer's protocol (ThermoScientific,USA). *E.coli* M15 cells were grown in 5 ml of LB broth overnight the day before transformation, 100 µl *E.coli* M15 cells were seeded in 10 ml of LB broth. T-solution was prepared by mixing about 250 µl of T-solution (A) and 250 µl of T-solution (B). About 75 µl of the overnight culture was added to 750 µl of C-medium. The culture was incubated at 37°C in a shaker incubator till O.D. reached about 0.4. The bacterial cells were centrifuged at 12000 rpm for 1 minute and the supernatant was discarded. The cells were resuspended in 150 µl of T-solution and incubated on ice for 5 minutes. Again centrifugation was done at 12000 rpm for 1 minute and supernatant was discarded. Finally, 50 µl of T-solution containing competent cells were transferred into microcentrifuge tube containing 7 µl of ligation mix and incubated on ice for 15 min. Then the transformed clones were plated onto LB agar containing Ampicillin (100 µg/ml) and Kanamycin (25 µg/ml). The LB agar plates were incubated at 37°C for the selection of the transformed clones based on antibiotic resistance.

3.2.11.3. Screening of recombinant clones

The recombinant clones harbouring plasmid DNA and insert were screened based on kanamycin (30 µg/mL) resistance for TatD Hydrolase and Hamolysin III in SUMO vectors, ampicillin (100µg/ml) resistance for Hamolysin III in pET32a vector and ampicillin (100µg/ml) and Kanamycin (30 µg/mL) resistance in pQE30Xa vector. After overnight incubation at 37°C, few colonies appeared on the LB agar plate. All colonies were picked and inoculated in 5 ml of LB broth containing appropriate antibiotic. The test tubes were kept at 37 °C for overnight in shaker incubator at 180 rpm.

3.2.11.4. Colony PCR screening for recombinants

Colony PCR was done using recombinant plasmid clones to amplify TatD Hydrolase gene insert using pRham-SUMO-TatD-For and pRham-SUMO-TatD-Rev primers. Recombinant clones obtained for Haemolysin III gene were also confirmed by the respective specific primers of different vectors.

3.2.12. Sequencing of the TatD Hydrolase recombinant clone and analysis

Recombinant plasmids were extracted from the overnight culture using Qiagen plasmid extraction kit (Catalogue no.- 27104) as per the manufacturer's instructions. 5 ml TatD Hydrolase overnight culture was pelleted by centrifugation at 12,000 rpm for 3 min at room temperature. The bacterial cells were resuspended in 250 µl of buffer P1 and transferred to the microcentrifuge tube. 250 µl of Buffer P2 was added and mixed thoroughly by inverting the tube 4-6 times. 350 µl Buffer N3was added and mixed immediately and thoroughly by inverting the tube 4-6 times. Centrifuged for 10 min at 12000 rpm and 800µl supernatant was added to the spin column by pipetting. It was again centrifuged for 1 min at 12000 rpm and the flow-through was discarded. 0.5 ml Buffer PB was added. Centrifuged for 1 min at 12000 rpm and the flow-through was discarded. 0.75 ml Buffer PE was added, centrifuged for 1 min at 12000 rpm and the flow-through was discarded. Again centrifuged for 1 min to remove the residual wash buffer. The column was then placed into a sterile 1.5 ml microcentrifuge tube. To elute the plasmid DNA 30 µl of Buffer EB was added, allowed to stand for 1 min and then centrifuged for 1 min at 12000 rpm.

pRham-N-His SUMO-Kan- *TatD Hydrolase* construct was sequenced at a custum DNA Sequencing facility (Eurofins). Sequences were analysed using DNASTAR software and subjected to analysis using BLAST (Basic Local Alignment Search Tool) programme of NCBI (National Centre for Biotechnology Information) against the nucleotide or protein database. A phylogenetic analysis using the nucleotide as well as the protein sequence of the gene was also done using the Mega6 software.

3.2.13. EXPRESSION AND PURIFICATION OF RECOMBINANT *TatD Hydrolase* and *Haemolysin III* genes

3.2.13.1. Induction of expression

Induction of expression in p-Rham-N-His SUMO Kan vector:

The recombinant clones were screened by colony PCR and the positive clone was grown in LB Kanamycin (30 µg/ml) broth at 37 °C with constant shaking at 160 rpm overnight. 50 µl of the overnight culture from each clone was inoculated into 5 ml of LB broth and further incubated at 37°C with constant shaking until OD_{600} reached 0.4-0.6. One ml of the culture

was collected from each tube and kept as uninduced control. The uninduced culture was pelleted and stored at -20 °C till use. To the rest of the culture, L-rhamnose was added to a final concentration of 0.2% and kept at 37 °C, with constant shaking at 180 rpm. One ml of the induced culture was collected after 12 hrs. All the induced cultures were pelleted by centrifugation at 8,000 rpm for 4 min in a microcentifuge (Eppendrof, Germany). The uninduced and the induced pelleted cells were suspended in 40µl of PBS pH 7.4 and 10 µl of 5X Laemmli buffer, kept at boiling temperature for 10 min and then stored at -20 °C for further use.

Induction of expression in pETite SUMO vector, pET32a vector and pQE30Xa vector:

For pETite SUMO vector, about 8 transformed colonies were randomly picked and inoculated in 10 ml of LB broth containing Kanamycin (30 µg/ml). For pET32a, about 8 transformed colonies were randomly picked and inoculated in 10 ml of LB broth containing Ampicillin (100 µg/ml). For pQE30Xa, about 8 transformed colonies were randomly picked and inoculated in 10 ml of LB broth containing Ampicillin (100 µg/ml) and Kanamycin (30 µg/ml). The test tubes were kept at 37°C with constant shaking at 160 rpm overnight. 50 µl of the overnight culture from each clone was inoculated into 5 ml of LB broth and further incubated at 37 °C with constant shaking until OD_{600} reached 0.4-0.6. One ml of the culture was collected from each tube and kept as uninduced control. The uninduced culture was pelleted and stored at -20 °C till use. To the rest of the culture 1 mM IPTG was added and kept at 37°C with constant shaking at 180 rpm. One ml of the induced culture was collected after 12 hrs. All the induced cultures were pelleted by centrifugation at 8,000 rpm for 4 min in a microcentifuge (Eppendrof, Germany). The uninduced and the induced pelleted cells were suspended in 40µl of PBS pH 7.4 and 10 µl of 5X Laemlli buffer, kept at boiling temperature for 10 min and then stored at -20 °C for further use. The culture was even induced with different concentration of IPTG- 0.5 mM, 1mM, 1.5 mM and 2 mM. The culture samples (1 ml) were collected at 3 hrs, 6 hrs and 12 hrs incubation after the induction with IPTG. Auto-induction method using 0.5% glocose and 1%, 2% and 3% ethanol was also done [Chettri *et al.*, 2015].

3.2.13.2. Confirmation of expression

(i) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was carried out as per the protocol described by Laemmli (1970) with slight modifications. The gel casting platform was assembled. The stacking (4.5%) and separating gels (12%) were prepared (Appendix). The separating gel was poured in between the glass plates and over layered with a thin film of n-butanol for formation of compact gel. The butanol was removed by flushing with distilled water before stacking gel was layered above the separating gel. The polymerized gel was transferred to an electrophoretic apparatus with buffer chamber filled with 1X Tris Glycine electrode buffer (Appendix). About 35 µl of cell lysate was mixed with equal volume of 2x sample loading buffer, heated at 95°C for 10 mins and then loaded into gel along with protein molecular weight marker. The gel was electrophoresed at 100 V till the tracking dye traversed the stacking gel. The voltage was then maintained at 75 V till the dye reached bottom of the separating gel. Subsequently, the gel was stained with Coomassie Brilliant Blue (CBB) staining solution (Appendix) for 2 -3 hrs and then destained with frequent changes of destaining solution (Appendix) till background was cleared. Gel with clear discrete protein bands was analyzed with the help of white light illuminator and photographed.

3.2.13.3. Large scale production of recombinant TatD Hydrolase protein

About 5 ml of overnight grown culture of *E. cloni* 10G cells possessing recombinant clones were inoculated in 500 ml LB broth containing 30 µg/ml of kanamycin and incubated at 37 °C in shaking incubator until the O.D. $_{600}$ reaches 0.4-0.6 followed by induction with 0.2% L- rhamnose. The expressed recombinant protein was harvested 12 hrs after induction by pelleting the bacterial cells by centrifugation at 8000 rpm for 4 mins.

3. 2.13.4. Purification of recombinant TatD Hydrolase protein

The polyhistidine (6X His) tagged fusion protein was purified under denaturing conditions in urea using Ni-NTA affinity chromatography as per the instructions of the manufacturer (Qiagen, USA).

The culture was taken in 50 ml centrifuge tube and centrifugation done at 8,000 rpm for 15 mins and the bacterial pellet was stored at -20 °C. The pellet was thawed and resuspended in 9 ml of the lysis buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris base, pH- 8) (Appendix), mixed and incubated at 37 °C with shaking for 3 hrs for complete lysis. It was then sonicated at 10 Hz for 15 sec and 15 sec pause (15 cycles).

The sonicated bacterial pellet was centrifuged at 10,000 rpm for 15 mins at 4 °C. The supernatant was taken carefully without disturbing the pellet. Simultaneously Qiagen column was prepared by adding 3 ml of Nickel- NTA agarose bead. Ethanol was allowed to move out so that Nickel-NTA agarose remain in column. Then 30%, 50%, 70%, Absolute alcohol was allowed to pass through the column followed by 70%, 50%, 30% ethanol. Column was equilibrated with lysis buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris base, pH- 8) and supernatant was mixed with Nickel agarose slowly in lysis buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris base, pH- 8) and supernatant allowed to pass through atleast 3 times. The column was washed with wash buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris base, pH- 6.3) (Appendix) and the bound proteins were eluted with elution buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris base, pH- 4.5) (Appendix). 0.5 ml fraction of elute was collected in 1.5 ml, 10 microcentrifuge tube. The Nickel agarose column was washed with ethanol 30%, 50%, 70%, absolute alcohol followed by 70%, 50%, and 30% ethanol and stored at 4 °C.

3.2.14. Protein refolding and estimation

The purified protein was pooled in a dialysis membrane (Thermo Scientific) and dialyzed against decreasing concentrations of urea viz 7 M, 6 M, 5 M, 4 M, 3 M, 2 M, 1 M, 0.5 M urea and one litre phosphate buffered saline for overnight in order to remove urea. The protein concentration was determined by Bradford assay.

3.2.15. Quantification of purified protein

To determine total protein concentrations, the Bradford assay as described by Ramagli and Rodriquez (1985) was used. The Bradford assay, a colorimetric protein assay, is based on an absorbance shift in the dye Coomassie when the previously red form coomassie reagent changes and stabilizes into coomassie blue by the binding of protein. 1 mg/ ml bovine serum

albumin (BSA) protein is used to prepare calibration curves. Standard bovine serum albumin were prepared by dissolving 0.25 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml, 1.25 mg/ml bovine serum albumin were used as standards and accordingly purified protein concentrations were measured.

Concentration of protein was calculated by the formula:

Concentration of the protein = (optical density of test sample/ optical density of standard) × Concentration of standard.

3.2.16. Western blot for detection of the his tagged recombinant TatD Hydrolase protein

The reactivity of the recombinant protein with the antiserum raised against the recombinant protein was checked by Western blot (Towbin *et al*, 1979). About 10-20 µl of the purified recombinant protein was electrophoresed on a SDS-gel and subsequently transferred on to a nitrocellulose membrane using western blotting electrophoretic apparatus (BioRad, USA) after soaking the filter paper and NCM in transfer buffer (Appendix) for at least 5 minutes. The electrophoretic apparatus was set up.The filter papers were set and over them the gel was placed on the cathode side and the NCM over it. It was clamped and fitted in the electrophoresis tank filled with the transfer buffer (Appendix). Then current was applied at 22 mA and 70 volts for 70 mins. Transferred NCM was then placed in blocking buffer (Appendix) overnight at 4 °C. Membrane was washed 3 times with PBST for 5 minutes. Then 1: 200 dilution of primary antibody (Goat anti-chicken IgY) in blocking buffer was added and incubated at 37 °C for 2 hrs. After washing 3 times with PBST, 1:10,000 dilution of secondary antibody (anti-chicken HRPO conjugate) in blocking buffer was added and kept at 37°C for 2 hrs. It was again washed 3 times with PBST. Membrane was developed with developing solution DAB+H_2O_2 (Appendix) and the reaction was stopped by washing the nitrocellulose membrane with the distilled water.

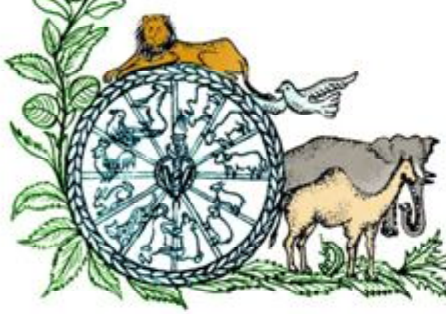
3.3 Functional characterization of Recombinant TatD Hydrolase

TOLUIDINE BLUE-O DNase ASSAY was prepared (Appendix). Toluidine blue DNase agar was poured onto the glass slide to a thickness of about 3 mm.Wells were punched after agar solidification and charged with the samples- 5µl of DNase 1 (positive control), 5µl of PBS (negative control), DNase 1(5µl) + TatD Hydrolase recombinant protein(10µg), TatD Hydrolase recombinant protein(10µg), TatD Hydrolase recombinant protein(10µg) + ATCC 10092 *Clostridium chauveoi* culture supernatant (5 µl) and ATCC 10092 *Clostridium chauveoi* culture supernatant (5 µl).The glass slides were then kept for incubation at 37°C for 5-6 hrs.

The culture supernatant taken for this experimental protocol was from the *C. chauvoei* culture of 12 hrs and then supernatant was collected from the culture by centrifuging it at 12000 rpm for 15 mins.

The diameter of zone formed around the charged wells was measured using a scale and hence the effect on the deoxyribonuclease activity was recorded

Results



4 RESULTS

4.1 Characterization of *C. chauvoei* culture

C. chauvoei culture was inoculated in ATCC 2107 media and incubated at 37°C under anaerobic conditions. Good growth was observed after 24-48 hrs.

4.1.1 Gram staining of the *C. chauvoei* culture

Gram staining of the culture revealed Gram positive, short, thick rods in a chain of 2-3 organisms (Fig.1).

4.1.2. Isolation of DNA from *C. chauvoei* culture

DNA was isolated from *C. chauvoei* culture by Genomic DNA extraction kit based extraction method. The integrity of DNA was checked by 1% agarose gel electrophoresis. Gel documentation analysis showed a strong DNA band. This genomic DNA was used as template for 16S-23S rDNA spacer and *cctA* gene specific PCR assay and amplification of TatD Hydrolase and Haemolysin III gene.

4.1.3. *C. chauvoei* 16S-23S rDNA spacer region and *cctA* gene specific PCR

PCR confirmation of culture was done by using primers specific for 16S-23S rDNA spacer region (Sasaki *et al*., 2001) and *cctA* gene using *C. chauvoei* genomic DNA as template. Agarose gel electrophoresis analysis of the PCR product showed specific amplification, product length of 522 bp (spacer region) and *cctA* gene of 983 bp confirming the identity of *C. chauvoei* (Fig. 2).

4.2. Molecular characterization of the *tatD* and *hlyIII* genes

4.2.1. PCR amplification of *tatD* and *hlyIII* gene of *C. chauvoei*

The *tatD* and *hlyIII* genes of *C. chauvoei* were amplified by self-designed gene specific primers using *C. chauvoei* DNA as template. Amplified products were analyzed by 1.3% agarose gel electrophoresis. *tatD* showed an intense amplification at 780 bp (Fig. 3) as expected and used for cloning in pRham-N-His SUMO Kan vector. *hlyIII* gene amplified at 699 bp, 699 bp, 700 bp and 557 bp (Fig. 13, 16, 19, 23) size for cloning in pRham-N-His SUMO Kan vector, pETite-N-His SUMO Kan vector, pET32a vector and pQE30Xa vector respectively. The PCR amplified *tatD* and *hlyIII* genes were eluted using gel extraction kit. The yield of the eluted DNA was approximately 80% of the original PCR product.

4.3. Cloning of *tatD and hlyIII* genes of *C. chauvoei*

The PCR products having the SUMO flanking region were ligated with pRham N-His SUMO Vector, transformed into *E. cloni* 10G cells, plated on LB kanamycin agar and incubated overnight at 37°C. LB kanamycin agar plate showed only around 4 colonies for *tatD* and 2 colonies for *hlyIII*. For *hlyIII* in case of pETite-N-His SUMO Kan vector, pET32a vector and pQE30Xa vector 15,19 and 8 colonies were visible respectively. The colonies were inoculated into 5 ml LB broth containing the appropriate antibiotic. LB broth containing single colony was kept for overnight growth in shaker incubator at 37°C and 160 rpm. pRham N-His- SUMO – *tatD* and *hlyIII* clones were then confirmed by colony PCR, which showed amplicons of desired sizes of 780 bp and 699 bp respectively (Fig. 4 and Fig. 14). Clones of *hlyIII* in pETite-N-His SUMO Kan vector, pET32a vector and pQE30Xa vector amplified at the expected size of 699 bp, 700 bp and 557 bp respectively (Fig. 17, Fig. 20, Fig. 24).

Plasmid was isolated from *tatD* clones and sequenced using sequencing primers specific for the vector backbone. Sequence analysis confirmed that the clones were indeed positive for *tatD* gene. The sequence obtained was subjected to BLAST analysis which revealed its maximum similarity to the *Clostridium chauvoei* JF4335 genome assembly (Fig. 5). The sequence was submitted to NCBI nucleotide sequence database and was assigned with accession number - MF177720. The nucleotide as well as the protein sequence for the *tatD*

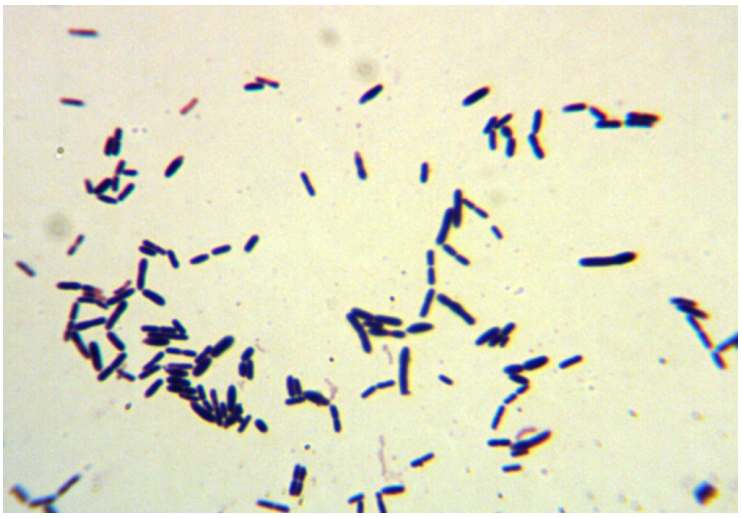


Fig. 1: Gram staining of *C. chauvoei* vegetative cells: Gram positive, short thick rods in a chain of 2-3 organisms

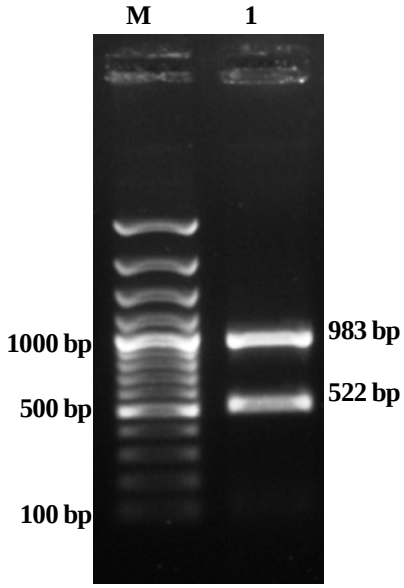


Fig. 2: Confirmation of *C. chauvoei* (ATCC 10092) by PCR based on 16S-23S rDNA spacer gene and *cctA* gene
Lane M : 100 bp DNA ladder
Lane 1 : PCR product

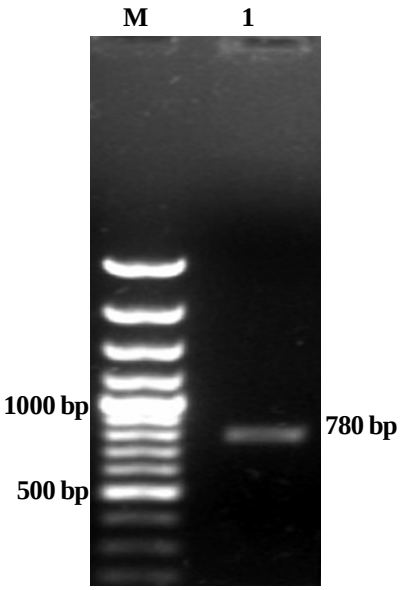


Fig. 3: PCR amplification of *tatD* gene of *C. chauvoei*
Lane M : 100 bp DNA Ladder
Lane 1 : *tatD* gene

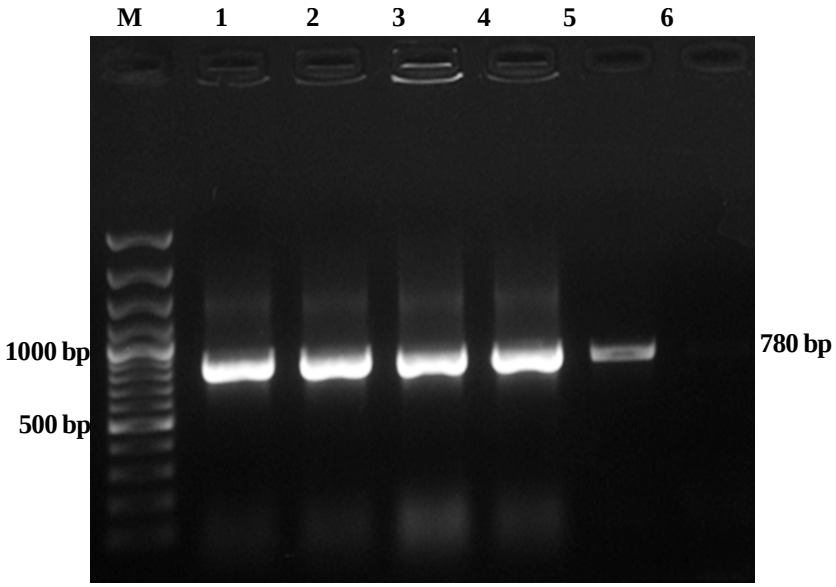


Fig. 4: Confirmation of the recombinant clones by colony PCR
Lane M : 100 bp DNA Ladder
Lanes 1-5 : *TatD Hydrolase* amplicon of 780 bp size
Lane 6 : Negative control

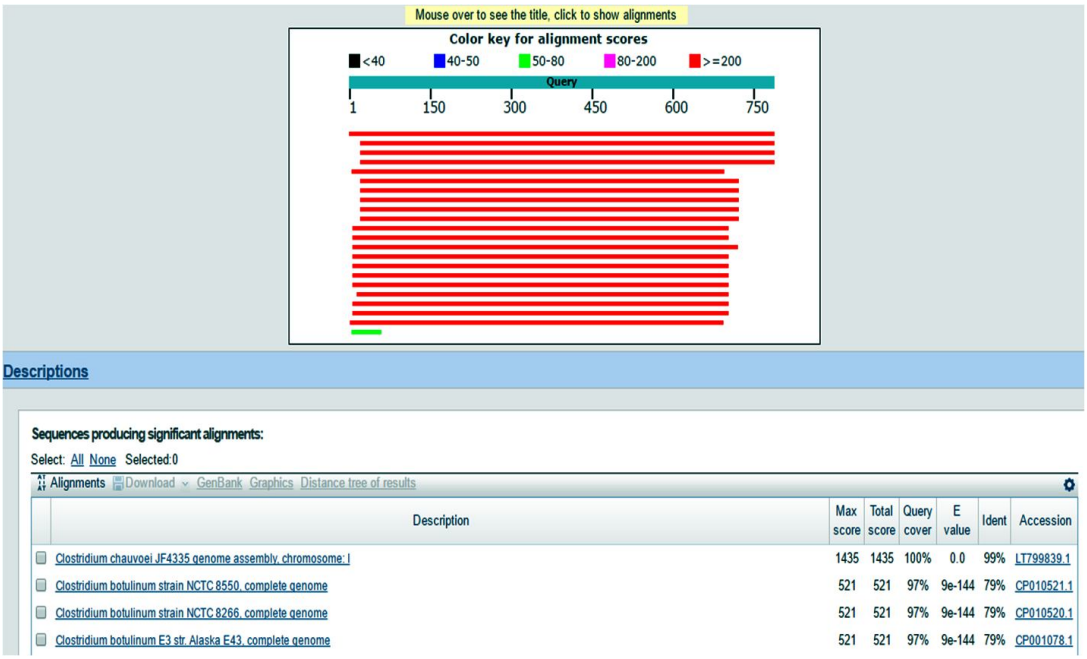


Fig. 5: BLAST analysis of rTatD sequence

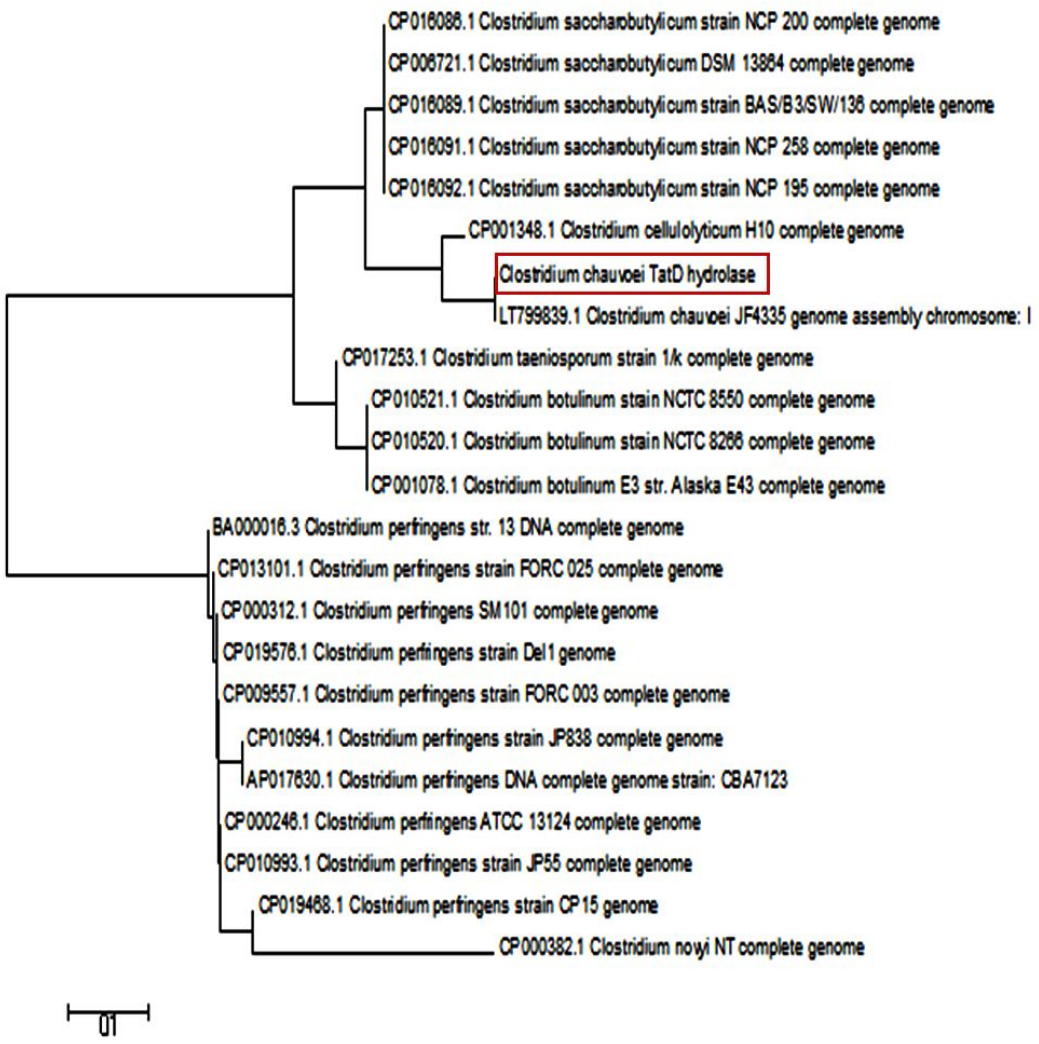


Fig. 6: Phylogenetic analysis of the *tatD* gene nucleotide sequence

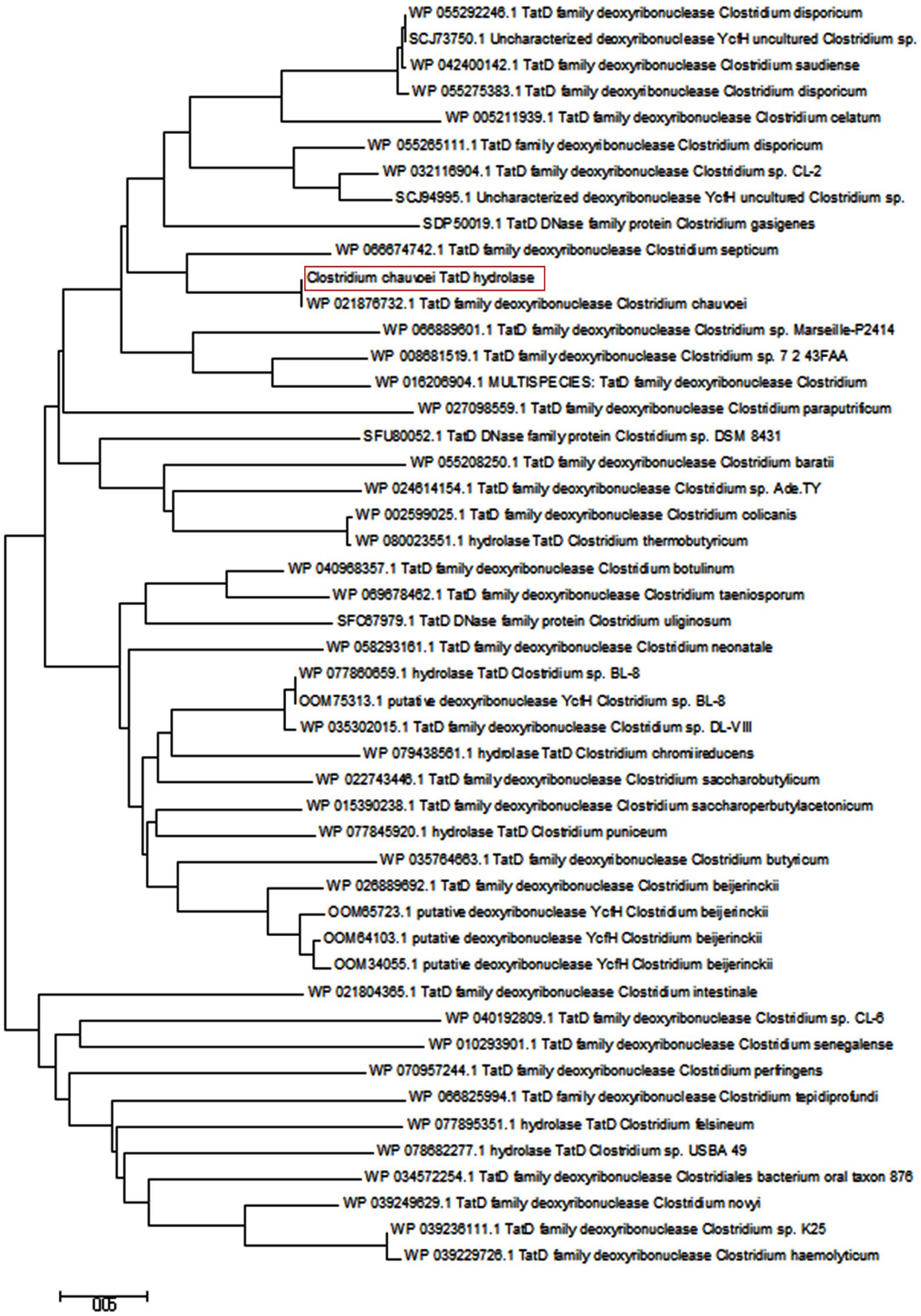


Fig. 7: Phylogenetic analysis of the rTatD Hydrolase protein sequence

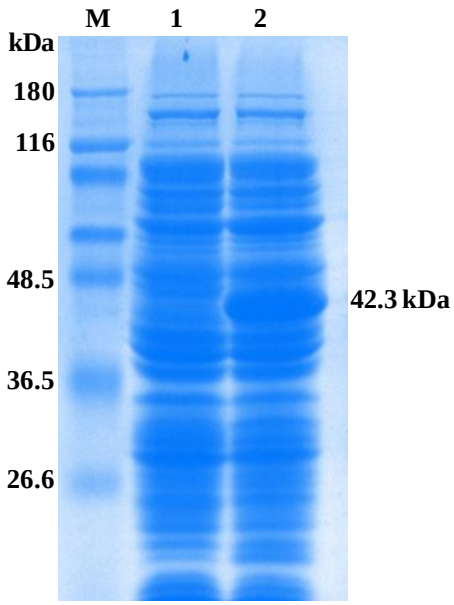


Fig. 8: Confirmation of overexpression of rTatD Hydrolase by SDS-PAGE analysis

Lane M : Prestained protein marker
Lane 1 : Uninduced cell lysate
Lane 2 : Overexpressed clone on Rhamnose induction

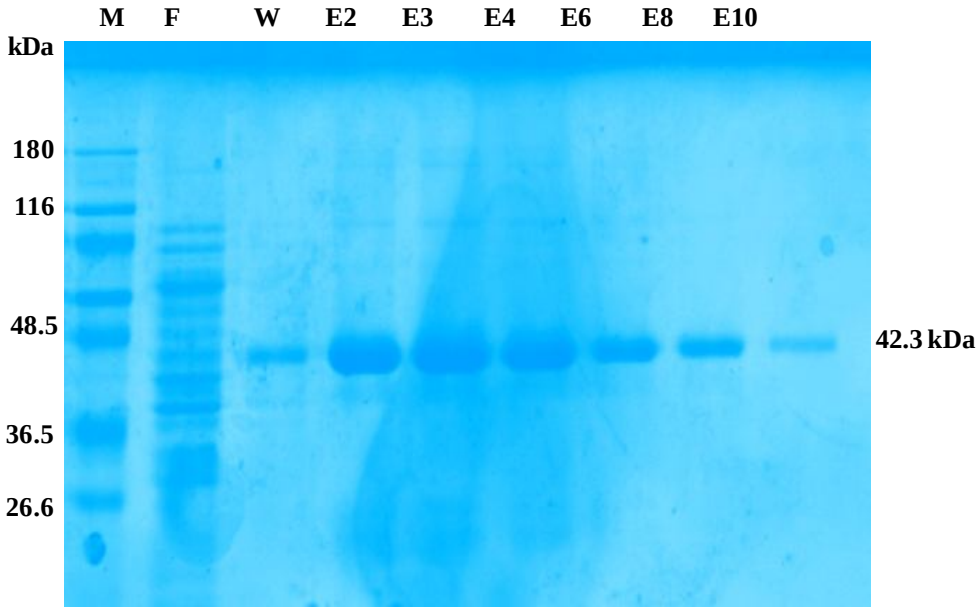


Fig. 9: SDS-PAGE Profile of purified His-tagged recombinant TatD Hydrolase

Lane M : Prestained protein marker
Lane F : Flowthrough fraction
Lane W : Wash fraction
E 2 to E10 : Different fractions of elutes

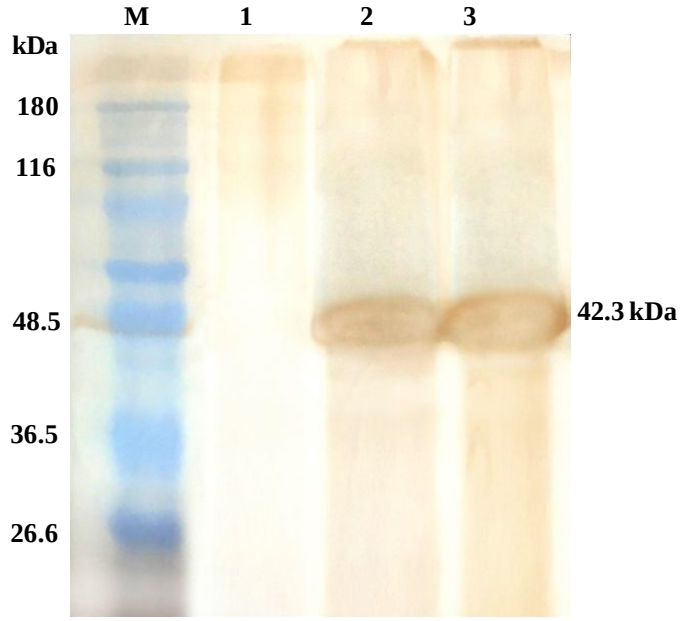
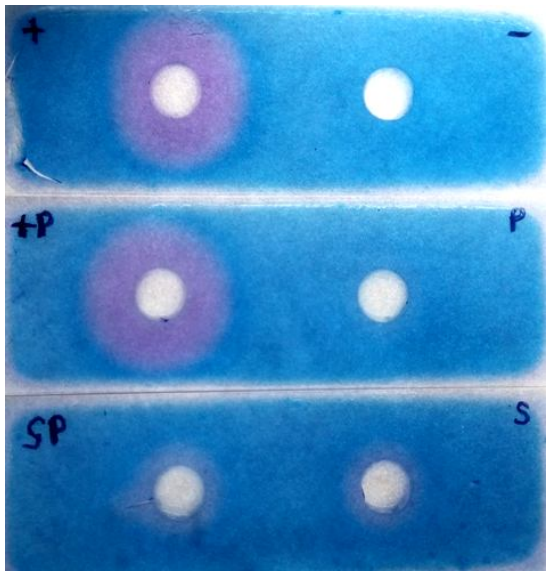


Fig. 10: Confirmation of overexpression of rTatD Hydrolase by Western blot; Lane
M : Prestained protein marker
Lane 1 : Uninduced cell lysate
Lane 2 : Induced cell lysate
Lane 3 : Purified rTatD Hydrolase



DNase I-positive control (5µl)

PBS- Negative Control (5µl)

DNase 1(5µl) + TatD Protein (10 µg)

TatD Protein (10 µg)

Culture Supernatant (5µl)+ TatD protein (10 µg)

Culture Supernatant (5µl)

Fig. 11: Determination of Toluidine Blue-O DNase assay of recombinant TatD Hydrolase protein

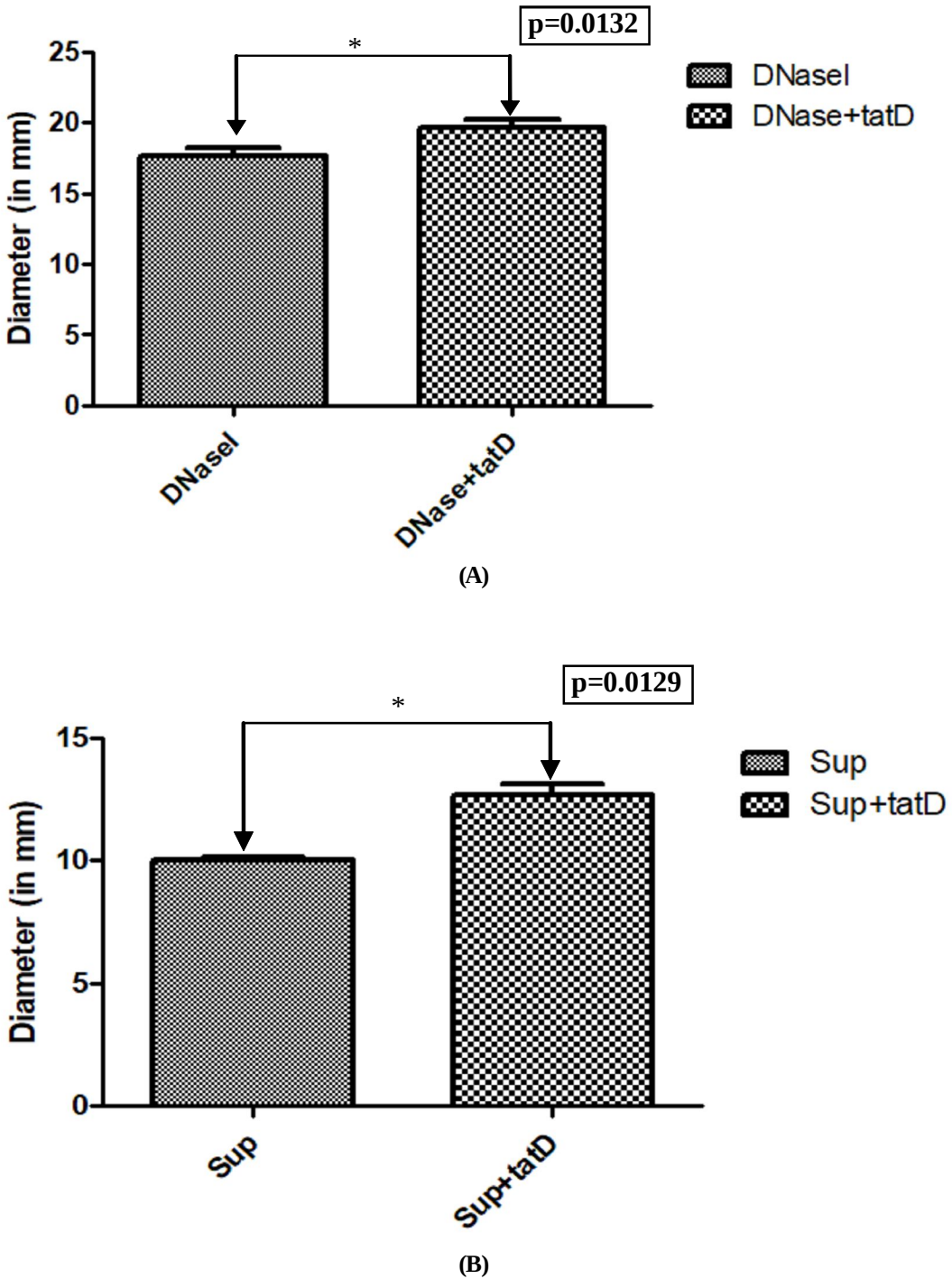


Fig. 12: Graphs showing the fold increase in the deoxyribonuclease activity by the rTatD Hydrolase protein with respect to DNase I enzyme (A) and *C. chauvoei* culture supernatant (B)

gene were subjected to phylogenetic analysis using the Mega6 software (Fig. 6 and Fig. 7). *tatD* nucleic acid sequence was most closely related to *Clostridium chauvoei* JF4335 genome assembly chromosome and the amino acid sequence was most closely related to the TatD family deoxyribonuclease *Clostridium chauvoei* . The sequence was also related to the sequences for deoxyribonuclease YcfH *Clostridium* sp.

4.4. Expression of the genes

4.4.1 Expression of the *tatD* gene of *C. chauvoei*

pRham N-His-SUMO–*tatD* clones were grown in LB kanamycin broth till the O.D. reached 0.4, and then induced with L-Rhamnose to the final concentration of 0.2%. The uninduced and the induced cultures were collected and run in a 12% SDS- PAGE. When the sample buffer reached the lower end, gel was removed from cassette and kept for staining for 1-2 hrs. Next, the gel was kept for destaining overnight, TatD Hydrolase protein expression was observed at the level of 42.3 kDa (Fig. 8) as expected.

4.4.2. Expression of the *hlyIII* gene of *C. chauvoei*

The *hlyIII* gene expression was tried using four different vectors. It was done in the pRham-N-His SUMO Kan vector. The clones were induced with 0.2% L-Rhamnose and SDS-PAGE analysis was done, the expression of the protein was not observed. Then it was even tried by second set of primer. In this case also the clone obtained after the transformation was found to be positive but after induction of the culture no expression of the rHly-III was obtained. It was even tried by the auto-induction method using 0.5% Glucose but still no effect (Fig.15). Then it was tried in pETite-N-His SUMO Kan vector. It was induced with 1 mM IPTG. The induced culture was collected after 3 hrs, 6 hrs and 12 hrs of incubation. But in none of the samples protein expression was observed. Auto induction method with 0.5% Glucose was also done but no protein expression was observed (Fig. 18). It was tried in pET32a vector. The culture was induced with 1 mM IPTG. The induced culture was collected after 3 hrs, 6 hrs and 12 hrs of incubation. The culture was even induced at different concentrations of IPTG- 0.5 mM, 1 mM, 1.5 mM and 2 mM. The autoinduction method was even tried by adding 0.5% Glucose to the culture and also by adding 1%, 2% and 3% ethanol

to the culture. But all the above methods did not revealed the Hly-III protein expression (Fig.21, 22). It was tried in pQE30Xa vector. The culture was induced with 1 mM IPTG. The induced culture was collected after every 3 hrs upto 12 hrs of total incubation. The auto-induction method was also tried by adding 0.5% Glucose to the culture. But none of them gave the Hly-III protein expression (Fig.25).

4.5. Purification of expressed His-tagged TatD Hydrolase recombinant protein

Recombinant TatD Hydrolase protein was purified under denaturing conditions in urea at different pH by Ni-NTA chromatography using standard protocol. The efficiency of purification was assessed by SDS-PAGE electrophoresis of the eluted fraction. After staining and destaining of the PAGE gel, a very intense band of 42.3 kDa protein was observed corresponding to the size of recombinant TatD Hydrolase (Fig. 9).

4.6. Confirmation of expression and purification of r*TatD Hydrolase* protein by Western blotting using anti histidine antibodies.

Furthermore, the confirmation of recombinant *TatD Hydrolase* protein was done by western blotting using Goat anti-chicken IgY anbtibody, which showed a specific band of 42.3 kDa, while uninduced culture did not show any reaction (Fig. 10).

4.7. Protein concentration determination by Bradford assay

Bradford assay was done for determining protein concentration. Optical density was measured at 595 nm using Bovine Serum Albumin as standard and it was found that recombinant *TatD Hydrolase* protein concentration was about 0.819 mg/ml.

4.8. Functional characterization of recombinant TatD Hydrolase protein

Toluidine Blue-O DNase assay was done to check the deoxyribonuclease activity of the recombinant TatD Hydrolase protein (Fig.11). A statistical analysis of the DNase activity of the rTatD Hydrolase in the presence of the DNase I enzyme or *C. chauvoei* culture supernatant revealed that the protein showed a significant effect in the DNase activity as compared to DNase I or *C. chauvoei* culture supernatant alone (Table 5 and 6; Fig.12).

Table 5. Readings of different experiments of TBO DNase assay with DNase I taken as the positive control

DNase I	Dnase I + rTatD Hydrolase protein
18 mm	19 mm
18 mm	20 mm
17 mm	20 mm
Mean ± S.D.= 17.667 ± 0.577	Mean ± S.D.= 19.667 ±0.577
STD. Error mean = 0.33	STD. Error mean= 0.33
t TEST VALUE = 4.243*	p value = 0.0132 *(significant
at 5% level of significance)	

Table 6. Readings of different experiments of TBO DNase assay with *C. chauvoei* culture supernatant taken as the positive control

Culture supernatant	Culture supernatant + rTatD hydrolase protein
10.1 mm	12.4 mm
10.0 mm	13.0 mm
Mean ±S.D.= 10.05 ± 0.07	Mean ±S.D.= 12.7 ± 0.424
STD. Error mean= 0.05	STD. Error mean= 0.30
t TEST VALUE = 8.713*	p value = 0.0129 *(significant at 5% level of significance)

For expression of *hlyIII* gene in pRham SUMO-Kan vector

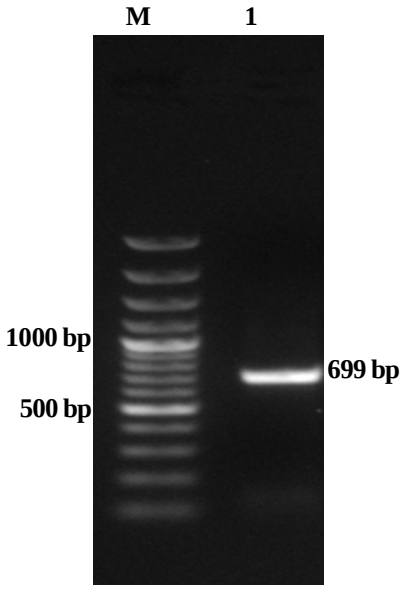


Fig. 13: PCR amplification of *hlyIII* gene of *C. chauvoei* Lane
M : 100 bp DNA Ladder
Lane 1 : *hlyIII* gene

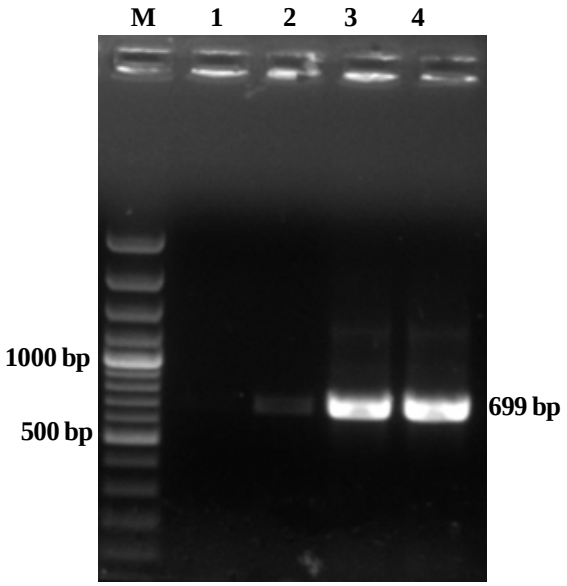
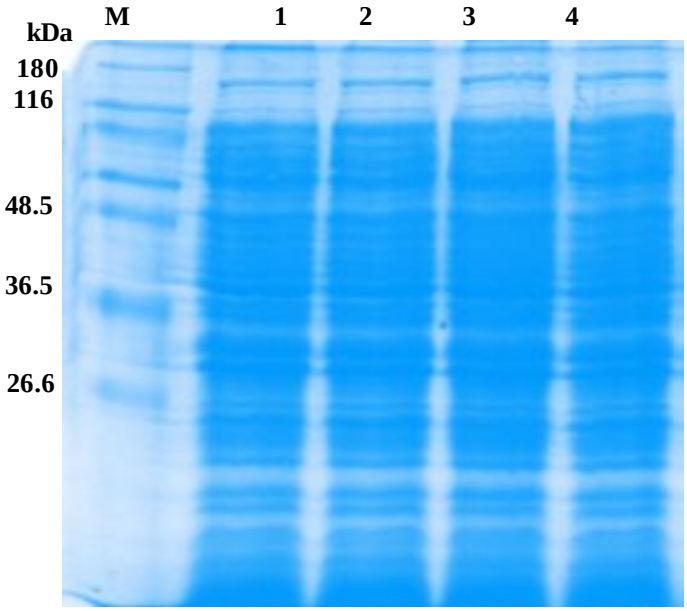
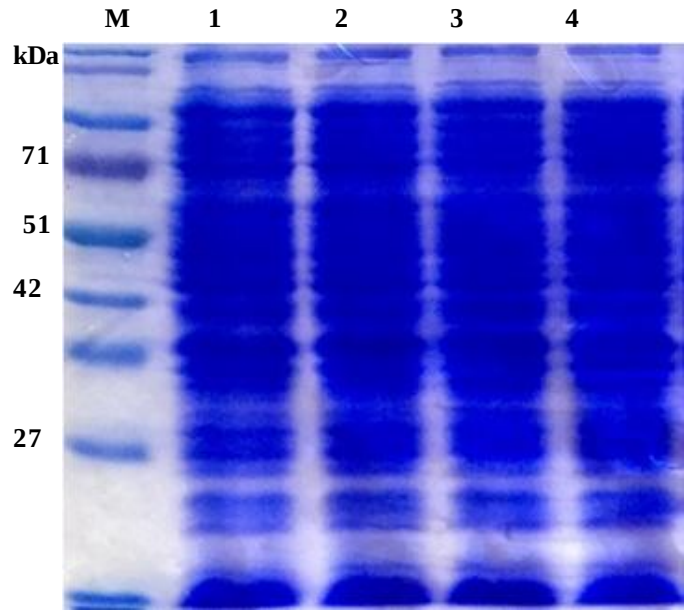


Fig. 14: Confirmation of the recombinant clones by colony PCR

Lane M : 100 bp DNA Ladder
Lane 1 : Negative control
Lanes 2- 4 : *hlyIII* gene



(A)



(B)

Fig. 15: Expression of *hlyIII* gene by SDS-PAGE analysis (A) usual method and **(B)** Autoinduction method

Lane M : Prestained protein marker

Lane 1 : Uninduced cell lysate

Lanes 2-4 : Overexpressed clones on Rhamnose induction

For expression of *hlyIII* gene in peTite SUMO vector

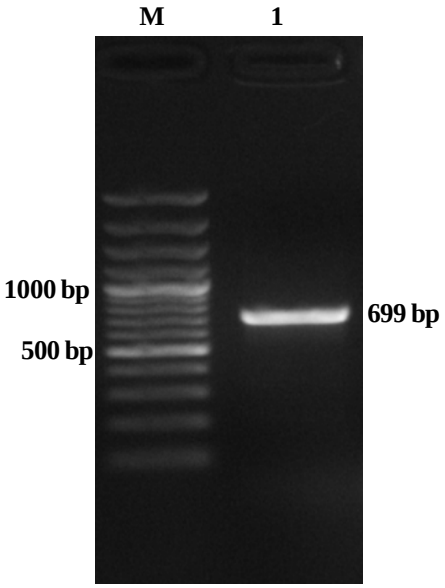


Fig. 16: PCR amplification of *hlyIII* gene of *C. chauvoei* Lane
M : 100 bp DNA Ladder
Lane 1 : *hlyIII* gene

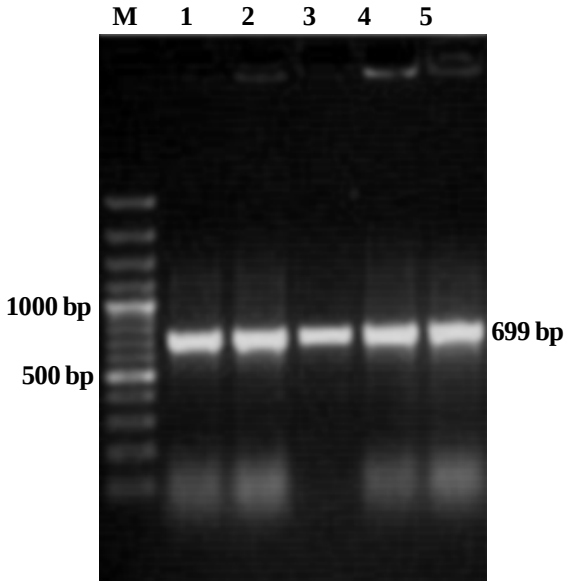


Fig. 17: Confirmation of the recombinant clones by colony PCR Lane
M : 100 bp DNA Ladder
Lanes 1-5 : *hlyIII* gene

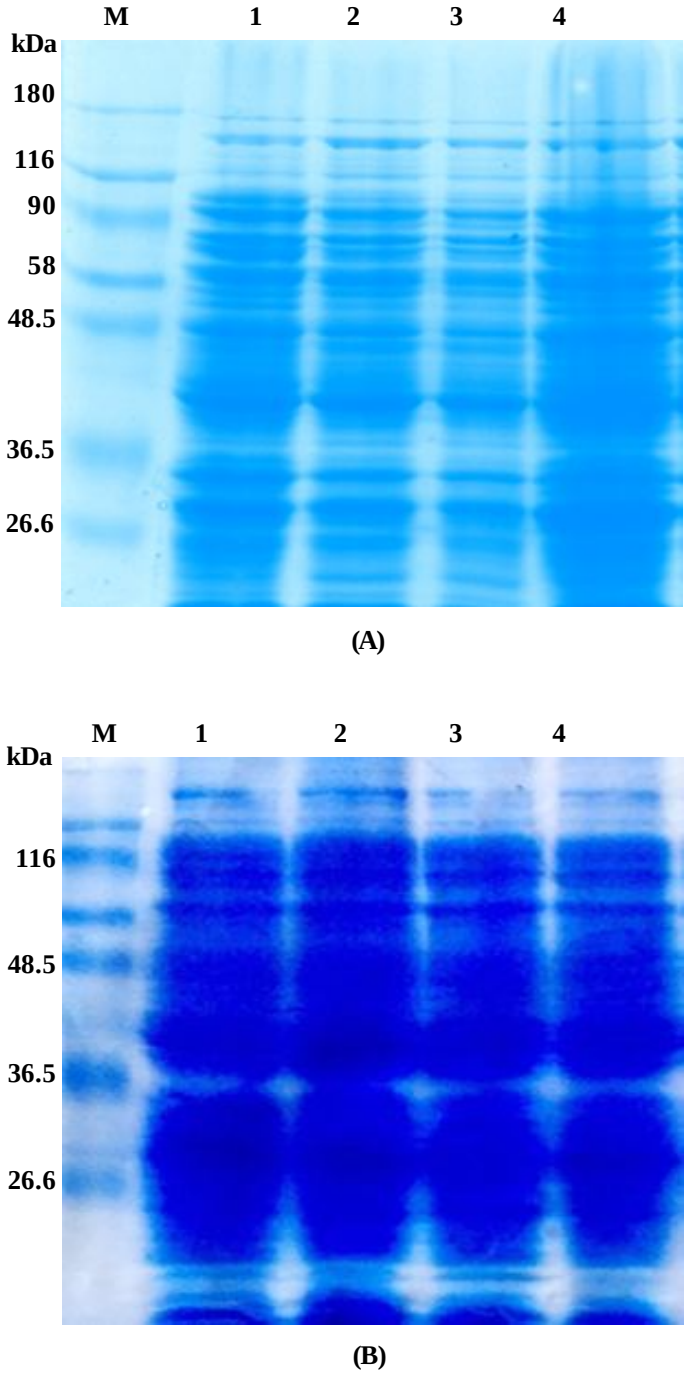


Fig. 18: Expression of *hlyIII* gene by SDS-PAGE analysis (A) usual method and (B) Autoinduction method

Lane M : Prestained protein marker
Lane 1 : Uninduced cell lysate
Lane 2 : Clones on IPTG induction after 3 hrs incubation
Lane 3 : Clones on IPTG induction after 6 hrs incubation
Lane 4 : Clones on IPTG induction after 12 hrs incubation

For expression of *hlyIII* gene in pET32a vector

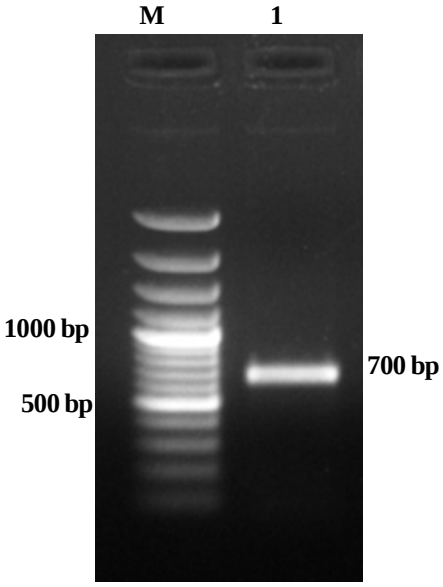


Fig. 19: PCR amplification of *hlyIII* gene of *C. chauvoei* Lane
M : 100 bp DNA Ladder
Lane 1 : *hlyIII* gene

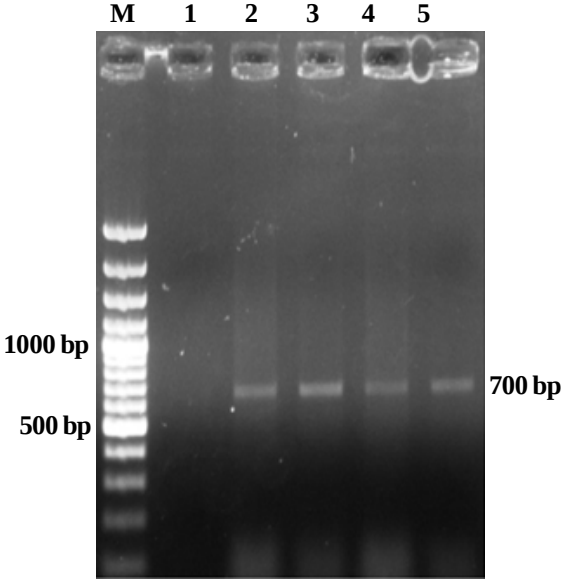


Fig. 20: Confirmation of the recombinant clones by colony PCR
Lane M : DNA Ladder
Lane 1 : Negative control
Lanes 2-5 : *hlyIII* gene PCR product

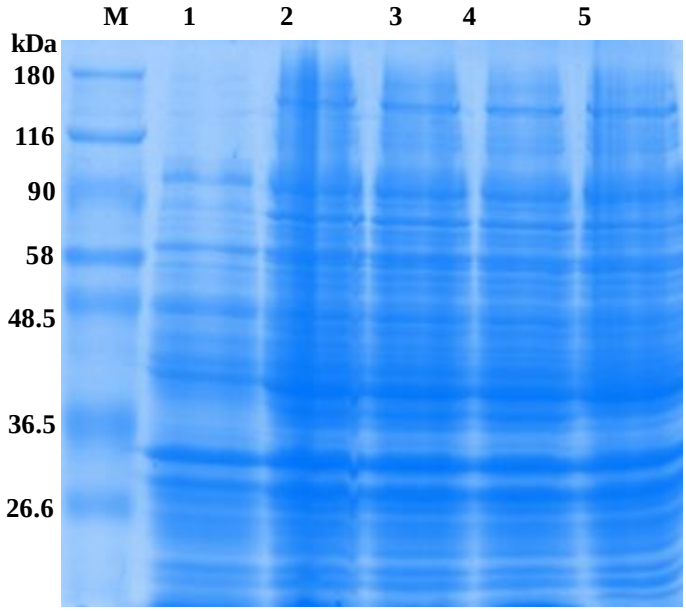


Fig. 21: Expression of *hlyIII* by SDS-PAGE analysis
Lane M : Prestained protein marker
Lane 1 : Uninduced cell lysate
Lanes 2 -5 : Clones on IPTG induction

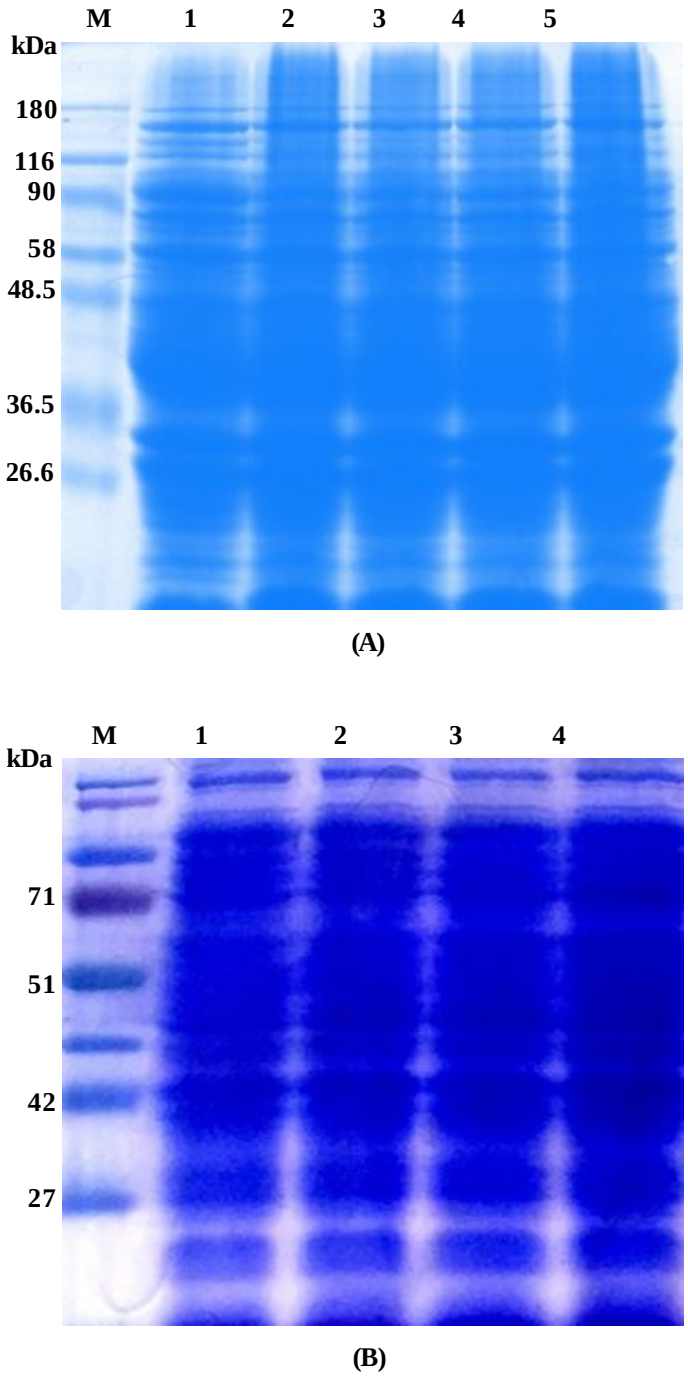


Fig. 22: Expression of *hlyIII* by SDS-PAGE analysis- (A) at different concentrations of IPTG (0.5 mM, 1mM, 2mM, 2.5mM); **(B)** Autoinduction method

For expression of *hlyIII* gene in pQE30Xa vector

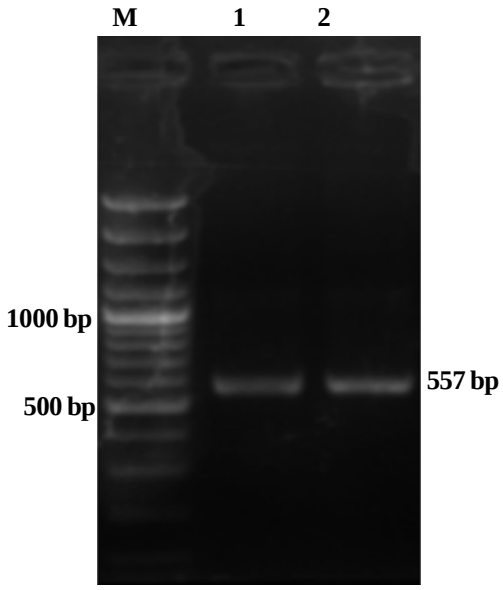


Fig. 23: PCR amplification of *hlyIII* gene of *C. chauvoei*
Lane M : 100 bp DNA Ladder
Lanes 1-2 : *hlyIII* gene

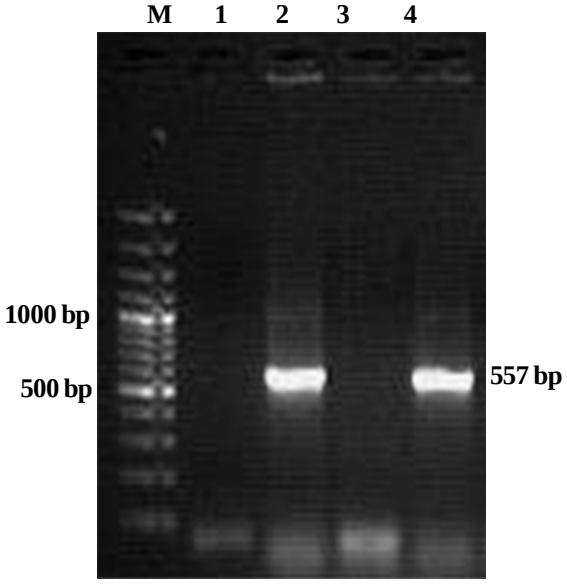
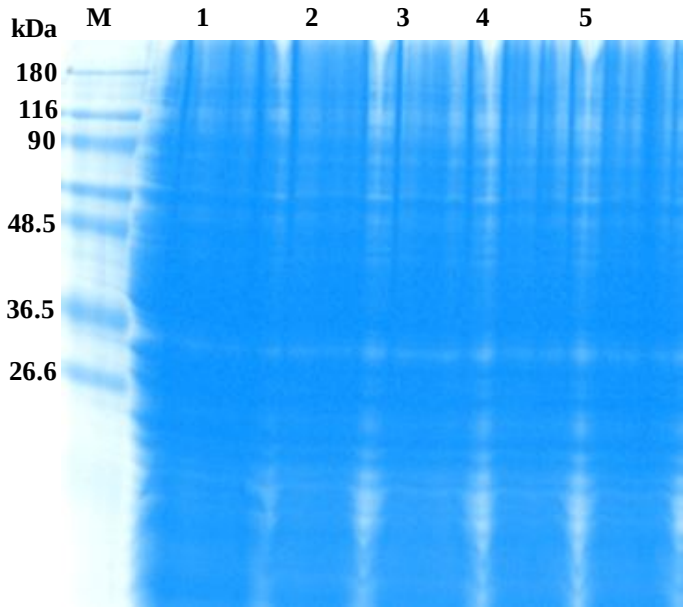
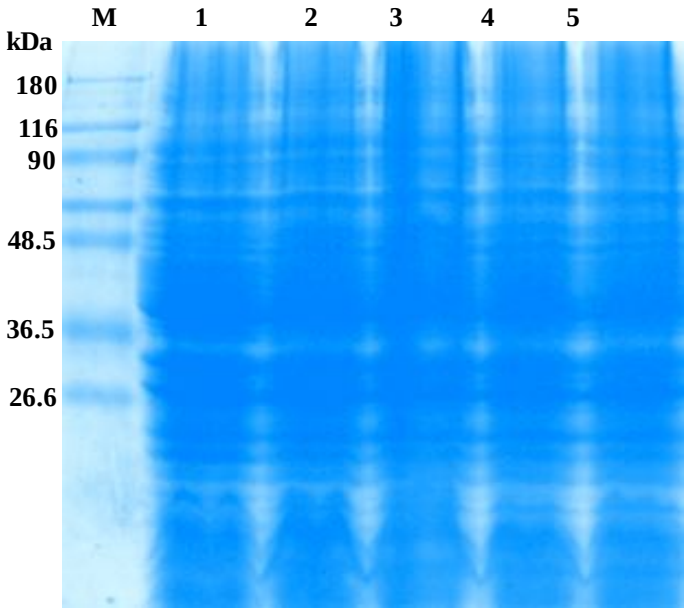


Fig. 24: Confirmation of the recombinant clones by colony PCR
Lane M : DNA Ladder
Lanes 1,3 : Negative control
Lanes 2,4 : *hlyIII* gene PCR product



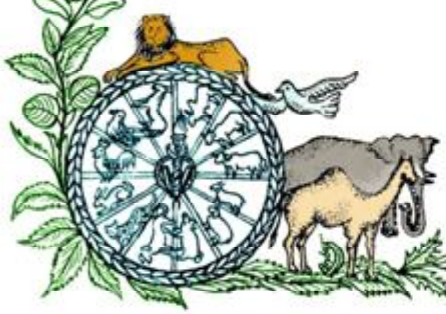
(A)



(B)

Fig. 25: Expression of *hlyIII* by SDS-PAGE analysis: (A) normal method; **(B)** Autoinduction method

Discussion



5 DISCUSSION

The largest barrier in the growth of livestock sector is the prevalence of various diseases like Foot and Mouth Disease (FMD), Peste des Petites Ruminants (PPR), Brucellosis, Anthrax, Hemorrhagic Septicemia (HS), Black Quarter (BQ), Classical Swine Fever (CSF), Ranikhet Disease (RD), Avian Influenza (AI) etc., which causes both heavy morbidity and mortality and consequently production losses affecting the animal productivity [Anonymous, 2017]. BQ is a soil-borne clostridial infection of bovines and is ranked fourth on basis of economic importance of the disease of livestock in India. It frequently occurs in the states of Andhra Pradesh, Telangana and Karnataka. The survival of clostridial spores in different soil types is not well understood and there seems to be some relationship between the soil type and number of rainy days for BQ to precipitate in a given location. Frequent soil contamination with infective spores from the poor disposal of the carcass adds to increased soil infection [Ahuja *et al*, 2008]. Black Quarter is caused by the bacteria, *Clostridium chauvoei*, a strictly anaerobic bacillus. Chabert (1782) was first to recognize *C. chauvoei* as a causative agent for Black Quarter disease, affecting cattle and sheep (Scott, 1924). Willis (1884) described *C. chauvoei* as a Gram positive, rod-shaped, strictly anaerobic, spore forming, non-capsulated and motile organism with straight and slightly curved axis.

It has been postulated that *C. chauvoei* produces four major toxins, viz., oxygen stable haemolysin (alpha toxin), deoxyribonuclease (beta toxin), hyaluronidase (gamma toxin) and oxygen-labile haemolysin (delta toxin) as well as sialidase and flagellin [Smith and Williams, 1984; Tamura *et al*., 1995], which are proposed to be the major contributors to the pathogenicity

of *C. chauvoei*. The genome of *C. chauvoei* reveals two different hyaluronidase genes [Frey and Falquet, 2014]. A sialidase activity has been reported in *C. chauvoei* [Moussa *et al*., 1958]. The flagella of C. *chauvoei* are involved in inducing immune resistance mechanisms, as demonstrated by the mouse protection test [Tamura *et al.*, 1984]. The flagella of *C. chauvoei* are of special interest for two reasons: first, they seem to be protective antigens [Tamura *et al*., 1984]; second, the flagellum of *C. chauvoei* is a virulence factor [Tamura *et al*., 1995]. Vilei *et al*. (2011) analyzed the sialidase activity of the NanA protein of *C. chauvoei* and cloned the sialidase gene *nanA*. CctA was shown to be a major haemolysin and protective antigen of *C. chauvoei* [Frey *et al*., 2012]. Among these, the beta toxin gene (deoxyribonuclease) is one of the major toxin responsible for the toxicity of *C. chauvoei*. Since the toxins of *C. chauvoei* are mainly responsible for its pathogenecity, their identification and characterization is very essential.

Though the deoxyribonuclease activity of the organism has already been made evident (Chaudhari and Singh, 1992), but the whole genome sequence of *C. chauvoei* available at the NCBI database do not reveal any specific sequence for the deoxyribonuclease gene. However when the DNase gene sequences from the other Clostridial species such as *C. butyricum* and *C. difficile* were analysed with the *C. chauvoei* genome, it showed similarities with the TatD Hydrolase (*tatD*) gene of *C. chauvoei*. TatD hydrolase is basically a protein present in the cytoplasm of the bacteria and has a magnesium dependent deoxyribonuclease activity. The DNase enzyme is responsible for the hydrolytic cleavage of phosphodiester bonds in the DNA backbone and therefore causing the degradation of the DNA. Timmis and Winkler (1973) observed the production of extracellular nuclease which degrade DNA preparation in *Clostridium* sp. *C. chauvoei* was found to possess extracellular cell wall compartmentalized as well as intracellular DNase.

hlyIII is also a toxin gene of *C.chauvoei* recently been stated by Frey *et al*. (2014). It is a pore-forming haemolysin with functional pore diameter of about 3-3.5 nm and is also shown to be present in most of the pathogenic bacteria including *Bacillus* species, where it is named as one of the haemolysin toxins [Baida and Kuzmin, 1996]. Though *cctA* is the major haemolysin involved in causing haemolysis, *hlyIII* may also contribute to the haemolysis. However, the role of this gene is not yet clearly established.

With all this background the present study was designed to clone and express *tatD* and *hlyIII* gene and their expression in the suitable vectors. Further the recombinant protein obtained to be utilized for the functional characterization of the genes.

The *C. chauvoei* culture was revived and characterized by cultural and molecular methods. On the Gram staining of the culture, Gram positive rods in short chains of 2-3 were observed. Some rods having sub-terminal spores were also visible. Then the culture was examined by molecular methods- confirmation by 16S-23S rDNA spacer region and *cctA* gene specific primers. Genomic DNA of the *C. chauvoei* culture was isolated. PCR amplification of *C. chauvoei* culture with 16S-23S rDNA spacer region primers was observed at 522 bp size and with *cctA* gene primers at 983 bp size as expected.

The *tatD* gene was amplified, purified and cloned into pRham N-His SUMO Kan Vector, transformed into *E. cloni* 10G cells and expressed into the prokaryotic expression system. The clones appeared as colonies on the LB agar plate containing Kanamycin which were confirmed by colony PCR. They gave amplification at the desired size of 780 bp with the TatD Hydrolase primers. The over expression of the gene was observed in 12 hrs of incubation after induction of the culture with 0.2% L-Rhamnose, when the O.D. reached 0.4. On SDS-PAGE analysis, the expressed protein band was observed at 42.3 kDa size. The plasmid was extracted from the culture of the clones and sequenced. On analyzing the sequence against the sequence database using the BLAST programme of NCBI, revealed its maximum similarity to the *Clostridium chauvoei* JF4335 genome assembly. The phylogenetic analysis of the TatD Hydrolase nucleotide as well as the protein sequence was done with all the other bacterial species and a phylogenetic tree was prepared for both the nucleotide and the protein sequence using Mega6 software which revealed that the TatD nucleic acid sequence was most closely related to *Clostridium chauvoei* JF4335 genome assembly chromosome and the amino acid sequence was most closely related to the TatD family deoxyribonuclease of *Clostridium chauvoei* . The sequence was closely related to deoxyribonuclease YcfH of *Clostridium* sp. indicating that TatD Hydrolase may have deoxyribonuclease activity.

The recombinant TatD Hydrolase protein was then purified using the Ni-NTA agarose beads under denaturing conditions and elution of the protein was done in the Urea elution buffer at a pH of 4.5. The purified protein was then allowed to refold in the decreasing molarity

of urea.The protein did not show precipitation as long as urea was present in the solution but as the protein was transferred to 100% PBS it started precipitating after 20-30 mins. It was immediately removed at this point as the protein tends to lose its functional properties after precipitation. The concentration of the protein was calculated by the Bradford method and found to be 0.819 mg/ml. The purified rTatD Hydrolase protein was also confirmed by Western Blotting which revealed a thick clear band at 42.3 kDa size.

The rTatD Hydrolase protein was subjected to functional characterization by Toluidine Blue-O DNase assay. Though TatD protein did not show any DNase activity individually, it was shown to have a synergistic effect on the deoxyribonuclease activity. It increased the DNase activity when added along with DNase I and *C. chauvoei* ATCC 10092 culture supernatant. This indicated that *tatD* by itself may not be a true DNase, though it could be contributing to the DNase activity of *C. chauvoei*.

The cloning and expression of the *hlyIII* gene of *C. chauvoei* was tried in the pRham-N-His SUMO Kan vector, pETite SUMO vector, pET32a vector and pQE30Xa vector. The *hlyIII* gene amplified at 699 bp, 699 bp, 700 bp and 557 bp respectively for the above mentioned vectors. For pRham-N-His SUMO Kan vector cloning was done in *E. cloni* 10 G competent cells. The clones were obtained on LB agar containing 30 ìg/ml Kanamycin and were checked by colony PCR. The clones were positive by PCR. These were then subcultured and subjected to induction with 0.2% L-Rhamnose. On SDS-Page analysis no expression was observed in the induced culture samples. Auto-induction method in which 0.5% Glucose was added to the culture was also done, but still no protein expression.

For pETite SUMO vector the primers used were the same as in pRham-N-His SUMO Kan vector but in this after cloning in the *E. cloni* cells it has to be cloned in BL-21 cells. 10G cells were used for construction of clones in the pETite vectors. Their recA- endA- genotype allows recovery of high quality plasmid DNA. 10G Cells do not express T7 RNA polymerase and therefore were not used for expression from the pETite vectors. BL21 cells used for expression of cloned genes from the T7 promoter. These cells produce T7 RNA polymerase from the *lacUV5* promoter. BL21(DE3) cells produce high levels of Lac repressor protein from a specially engineered *lacI* gene. The abundant Lac repressor minimizes basal expression of T7 RNA polymerase within the host cells prior to induction. It also prevents premature

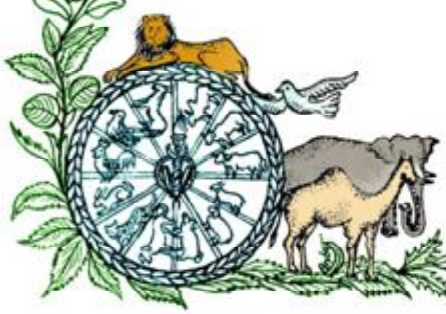
expression from the T7-lac promoter on the pETite vectors. This enhanced control over basal expression allows growth of clones that contain genes whose products might otherwise be toxic to the T7 host strain. The clones obtained were positive for the gene on checking with colony PCR but it also gave no expression on induction with 1 mM IPTG. A second set of primers was also designed for both SUMO vectors by exclusion of the signal sequence to ensure the gene expression but still no expression for the protein was observed.

In case of expression in pET32a, the cloning was done using BL-21 competent cells. The clones obtained were positive for the gene on checking with colony PCR but it also gave no expression on induction with 1 mM IPTG. For both pETite SUMO vector and pET32a vector, the induced culture was obtained after 3 hrs, 6 hrs and 12 hrs of incubation and it was also tried at different concentrations of IPTG- 0.5mM, 1 mM, 2mM and 2.5 mM but none of the efforts gave the *hlyIII* expression. In these auto-induction method was also done using both 0.5% glucose and 1%, 2% and 3% ethanol but this also did not gave expression of the protein.

In pQE30Xa vector, the transformation was done using Transformaid kit. The clones obtained were positive when checked by colony PCR and were induced with 1 mM IPTG. After induction the culture was collected at every 3 hrs interval for total 12 hrs but in none of them expression was observed. Auto- induction method using 0.5% Glucose was also done but it also gave no expression results.

Despite of all the above attempts for the expression of Haemolysin III gene we could not obtain the protein expression for it. All the above results may indicate that since Haemolysin III protein is a cytolysin and a pore-forming toxin, it may be very toxic for the host cells and as soon as it expressed itself, it might have caused lysis of the host cells and hence no expression could be observed on SDS-PAGE analysis.This observation is in concurrence with *Bacillus cereus*, in which the attempt for expression of *hlyIII* was carried out. However, they could not obtain it due to high toxicity of *hlyIII* for *E. coli* cells [Baida and Kuzmin, 1996]. Probably, similar mechanism could be happening in *C. chauvoei hlyIII* gene expression as well.

Summary and Conclusion



6 SUMMARY AND CONCLUSIONS

Relatively little research has been reported on the pathogenesis of *C. chauvoei* although the fatal disease "Black Quarter" (B.Q.) of sheep and cattle caused by this organisms is of considerable economic importance. B.Q. is a disease of global occurrence and hence its pathogenesis has to be studied keenly for its efficient diagnosis, treatment and control strategies. In a country like India, where livestock is present in abundance and is a major contributor of the country's economy the diseases like B.Q., which causes heavy losses of livestock is of high concern. It is a per acute bacterial disease caused by a Gram positive bacilli, *C. chauvoei* . The pathogenesis of *C. chauvoei* is dependent on the toxins it produce. Its major toxins postulated include: an oxygen-stable haemolysin and necrotizing factor (α), DNase (β), hyaluronidase (γ), oxygen labile haemolysin (δ), neuraminidase (sialidase), a surface adhesion protein, flagellin and *cctA* haemolysin. Among these, Deoxyribonuclease is one of the important toxins of the bacteria. The DNase activity of the toxin is responsible for the depolymerization of the DNA. The beta toxin of this bacteria is one of the least touched field and has to be characterized. On analysing the whole genome sequence of *C. chauvoei*, no gene was found to be coding for the DNase. Therefore the homology search of the whole genome sequence of *C. chauvoei* using the DNase genes of other Clostridial species was done and it was found that this DNase gene could show significant similarity with the TatD Hydrolase (*tatD*) gene of *C. chauvoei*. Hence, we hypothesized that *tatD* gene of *C. chauvoei* could be responsible for the DNase activity of the bacteria. Hence this gene was selected for its further functional characterization.

Though it was known that *cctA* is major haemolysin of *C. chauvoei*, recently it has been stated that other haemolysins can be potential candidates responsible for the haemolysis

caused by the bacteria, with *hlyIII* being one of them [Frey *et al.,* 2014]. Hence a study was designed to characterize this *hlyIII* gene and get the information about its role in pathogenesis and the exact function of the gene.

The present study was designed to clone, sequence, express and functionally characterize the *tatD* and the *hlyIII* genes of *C. chauvoei*.

The *C. chauvoei* ATCC 10092 strain was characterized by Gram's staining and molecular methods like PCR of 16S-23S rDNA spacer region primers and *cctA* primers. Primers were designed for the amplification of the *tatD* and the *hlyIII* genes. PCR amplification for the *tatD* gene using pRham-SUMO-*tatD* For and Rev was obtained at 780 bp size.The amplified gene products were then cloned into the pRham-N-His SUMO Kan vector to produce the recombinant plasmid. The recombinant clones obtained on the plates were then confirmed by colony PCR using *tatD* gene specific primers which yielded the product size of 780 bp. Further these were confirmed by sequencing. The sequence was analysed against the sequence database using the BLAST programme with the available *tatD* sequence of *C. chauvoei* at the NCBI database. The phylogenetic analysis of the nucleotide as well as the protein sequence was also done using the Mega6 bioinformatics software. The positive recombinant clones were then subcultured in LB broth containing 30 µg/ml kanamycin and then induced with final concentration of 0.2% L-Rhamnose which revealed the overexpression of the protein at 42.3 kDa.

The expressed rTatD Hydrolase protein was purified under denaturing conditions in 8M Urea using Ni-NTA affinity chromatography. The recombinant protein was then allowed to renature by dialyzing it against the decreasing molarity of urea in autoclaved PBS (pH 7.4). After dialysis the protein concentration was then determined by Bradford assay and was found out to be 0.819 mg/ml. The recombinant protein was further confirmed by Western Blotting using Goat anti-chicken IgY as the primary antibody and this revealed the presence of specific band at 42.3 kDa size. This rTatD Hydrolase protein was then subjected to functional characterization by Toluidine Blue-O DNase assay. Through this we could conclude the the rTatD Hydrolase protein did not show the DNase activity alone by itself but had a synergistic action along with the DNase I enzyme or the *C. chauvoei* culture supernatant. On statistical analysis, the values obtained were found to be significant i.e. a significant difference was found

between the positive control and the test (DNase I or culture supernatant alone and in presence of *tatD*). This indicated that though TatD Hydrolase may not be a true DNase, it may contribute to its DNase activity of *C. chauvoei*.

The *hlyIII* gene expression was tried using four different vectors. Firstly it was done in the pRham-N-His SUMO Kan vector. The gene was amplified and a product size of 699 bp was obtained. The purified PCR product was then cloned into pRham-N-His SUMO Kan vector and the recombinant clones were obtained on the LB agar containing 30 µg/ml Kanamycin which were then screened and confirmed by colony PCR. The clones were found to be positive as they amplified at the expected size of 799 bp but when the culture of these clones was induced with 0.2% L-Rhamnose and SDS-PAGE analysis was done, the expression of the protein was not observed. Then it was even tried by changing the forward primer. This got amplified at 790 bp size. In this case also the clone obtained after the transformation was found to be positive but after induction of the culture no expression of the rHly-III was obtained. It was also tried by the auto-induction method using 0.5% Glucose but still no effect.

Secondly the Hly-III protein expression was tried in pETite-N-His SUMO Kan vector. In this, the purified PCR product (799 bp) has to be first transformed into *E.cloni* 10 G cells. The plasmid then extracted from the clones and transformed into BL-21 cells. The clones were found to be positive by colony PCR. It was then induced with 1 mM IPTG. The induced culture was collected after 3 hrs, 6 hrs and 12 hrs of incubation. But in none of the samples protein expression was observed.

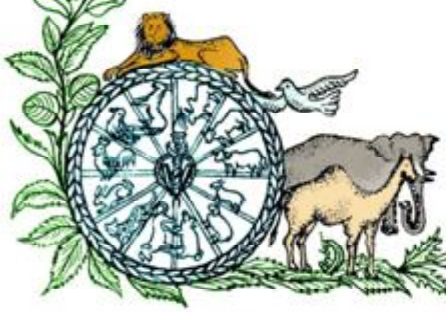
Thirdly, it was tried in pET32a vector. The vector plasmid as well as the PCR purified product were first digested with *Hind III* and *Nco I* restriction enzymes and then ligated in presence of T4 DNA ligase. The ligated product is then transformed into the BL-21 competent cells. The clones were obtained on the LB agar plates containing Ampicillin (100 µg/ml). On checking the clones by colony PCR all amplified at the expected size of 700 bp. Then the culture was induced with 1 mM IPTG. The induced culture was collected after 3 hrs, 6 hrs and 12 hrs of incubation. The culture was even induced at different concentrations of IPTG- 0.5 mM, 1 mM, 1.5 mM and 2 mM. The autoinduction method was also tried by adding 0.5% Glucose to the culture and also by adding 1%, 2% and 3% ethanol to the culture. But all the above methods did not reveal the Hly-III protein expression.

Fourthly, it was tried in pQE30Xa vector. The vector plasmid as well as the PCR purified product were first digested with *Hind III* and *Sac I* restriction enzymes and then ligated in presence of T4 DNA ligase. The ligated product is then transformed into M-15 cells. The clones were obtained on the LB agar plates containing Ampicillin (100 µg/ml) and Kanamycin (30 µg/ml). On checking the clones by colony PCR all amplified at the expected size of 557 bp. Then the culture was induced with 1 mM IPTG. The induced culture was collected after every 3 hrs upto 12 hrs of total incubation. The autoinduction method was also tried by adding 0.5% Glucose to the culture. But none of them gave the Hly-III protein expression.

The haemolysin gene expression was tried by all the possible methods. In case of each vector, the clone obtained was found to be positive for the gene when confirmed by the colony PCR, but when it was subjected further for expression either by 0.2% L-Rhamnose or 1 mM IPTG in the respective vectors, the Hly-III protein expression was not observed. The conclusion on the basis of all the evidence and reasoning of the results for *hlyIII* gene was that since the *hlyIII* gene is a cytolysin and a pore-forming haemolysin it may be toxic for the host cells. As soon as the gene was able to express itself, it might have caused the lysis of the *E. coli* cells and hence the protein expression was difficult to be obtained and observed in the SDS-PAGE analysis.

Since in our study we observed that *tatD* gene is not fully responsible for the deoxyribonuclease activity of *C. chauvoei*, therefore other genes could also be explored from the whole genome sequence of *C. chauvoei* which could have the deoxyribonuclease activity. In case of *hlyIII*, we could not obtain the protein expression in the different vectors by various different methods, therefore the attempt for its expression can be tried in various other expression systems.

Mini Abstract



7 MINI ABSTRACT

The pathogenesis of *Clostridium chauvoei* is mostly dependent on the activity of its toxins i.e to know the exact pathogenesis of B.Q., an efficient study of these toxins is foremost. Therefore, the present study was designed to target the two important toxin genes of the bacteria- TatD Hydrolase (*tatD*) and Haemolysin III (*hlyIII*). For both the genes the cloning and expression was attempted in suitable vectors and then their functional characterization was done. Primers were designed to amplify the genes. *tatD* was cloned and expressed in pRham-N-His SUMO Kan vector. It amplified at 780 bp size. The purified PCR product was transformed in pRham-N-His SUMO Kan vector DNA. Clones obtained were then cultured and induced with 0.2% L-Rhamnose. The protein got expressed at the expected size of 42.3 kDa. It was then purified, refolded and further confirmed by Western blotting. The tatD clones were sequenced and analysed against the sequence database using the BLAST programme with the available *tatD* sequence of *C. chauvoei* at the NCBI database. The phylogenetic analysis of the nucleotide as well as the protein sequence revealed maximum similarity with the *C. chauvoei* JF4335 genome assembly and TatD family deoxyribonucleases, respectively. The rTatD hydrolase protein was then functionally characterized by TBO DNase assay. By this a synergistic effect on the DNase activity was observed and the results obtained with respect to the DNase I enzyme or with *C. chauvoei* culture supernatant were found to be statistically significant. This indicated that though TatD Hydrolase may not be a true DNase, it may contribute to the DNase activity of *C. chauvoei*. The *hlyIII* gene amplified and cloned in pRham-N-His SUMO Kan vector, pETite-N-His SUMO Kan vector, pET32a vector and pQE30Xa vector respectively. Clones obtained were found to be positive and these were further subcultured and checked for the expression of the protein. The induction in case of pRham-N-His SUMO Kan vector was done by 0.2% L-Rhamnose. In case of pETite-N-His SUMO Kan vector, pET32a vector and pQE30Xa vector was done by 1 mM IPTG. But in all the above attempts for induction of Hly III protein the results were found to be negative. The expression was even tried by autoinduction method. Since everytime the clones obtained were positive but induction of expression got failed it was concluded that *hlyIII* may be toxic for the *E. coli* cells and hence it might have caused the lysis of the cells and its expression could not be obtained.

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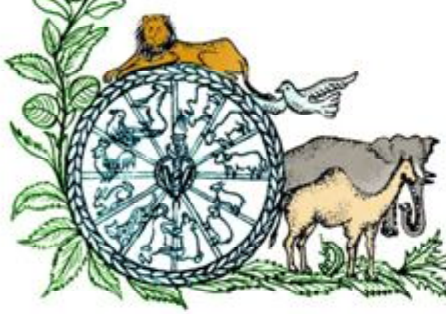

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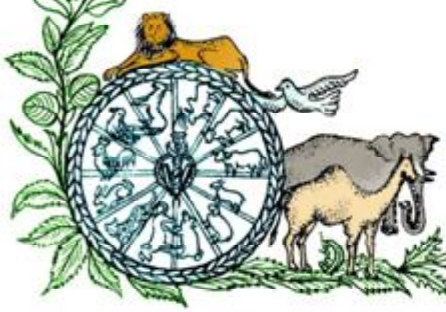

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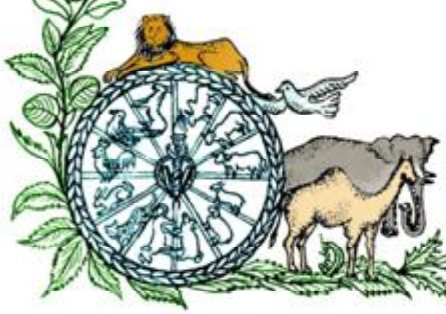
Appendix



APPENDIX

REAGENTS USED FOR BACTERIOLOGICAL PROCEDURES

1. ATCC 2107 Media

Tryptose	1 g
Beef extract	1 g
Yeast extract	0.3 g
Dextrose	0.5g
NaCl	0.5 g
Soluble Starch	0.1 g
L-cystine HCl	0.05 g
Na acetate	0.3 g

Distilled water upto 100 ml adjust the pH 6.8 with the 10 N NaOH. Autoclaving for 20 minutes at 15 psi on liquid cycle

2. LB (Luria-Bertani) broth

Bacto tryptone	10g
Bacto Yeast extract	5g
NaCl	10g
Deionized Water	950 ml

Adjust the pH to 7.0 with 5N NaOH.

Volume made up to 1L and sterilized by autoclaving at 15 psi (1.05Kg/cm2) for 20 minutes and the solution was stored at 4 ºC.

3. LB agar

1.5% Agar in LB broth media

Sterilized by autoclaving at 15 psi (1.05Kg/cm2) for 15 min and store at 4 ºC.

4. TSS Medium (For preparation of competent cells)- 50 ml

10% PEG	5 gm
5% DMSO	25 ml
20 mM $MgCl_2$	1 ml

Make upto 50 ml with LB

Filter through 0.22 µ syringe filters

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

1. Tris-acetate- EDTA (TAE) buffer (50X)

Tris base	242g
Glacial acetic acid	57.1ml
0.5 M EDTA (pH 8.0)	100ml

Distilled water was added to make the final volume upto 100 ml. A working solution of 1X was used.

2. Ethidium bromide stock solution (10mg/ml)

Ethidium bromide	100mg
Distilled water	10ml

The solution was mixed and stored at 4 ºC. A concentration of 0.5 µg/ml was used in preparing agarose gel.

3. DNA ladder marker (Working solution)

DNA ladder marker	1 part
6X loading dye	1 part
Nuclease free water	4 part

4. dNTPs (to be used in PCR reaction)

dATP	1 part
dGTP	1 part
dTTP	1 part
dCTP	1 part
Nuclease free water	6 part

5. 6X loading dye (100 ml)

Glycerol	60 ml
1M Tris Cl- (pH 8)	6 ml
0.5 M EDTA (pH 8)	1.2 ml
Bromophenol Blue	60 mg
Distilled water	32.8 ml

REAGENTS FOR CLONING & EXPRESSION

1. Kanamycin

Kanamycin powder	300mg
Sterile distilled water	10 ml

Filter sterilize and store at 4ºC

2. Ampicillin

Ampicillin powder	1 gm
Sterile distilled water	10 ml

Filter sterilize and store at 4ºC

3. Rhamnose 20%

Rhamnose	20g
Sterile distilled water	100 ml

Filter sterilize and store at 4ºC

4. IPTG (Isopropyl-β-D-thiogalactopyranoside) (1000 X)

IPTG	25 mg
Distilled water	1 ml

Sterilized by filteration. Stored at 4ºC

REAGENTS AND SOLUTIONS FOR SDS- PAGE

1. 30% Acrylamide- bisacrylamide mix

Acrylamide	30 g
Bis- acrylamide	0.8 g

Make volume upto 100 ml using DW. Mix by boiling and filter. Store at 4°C.

2. Ammonium persulphate (APS, 10% w/v)

Ammonium persulphate	100 mg
Distilled water	1 ml

Store at 4 °C and use within 7 days

3. 10% SDS

Sodium dodecyl sulphate	10 g
Distilled water	upto 100 ml

4. Resolving gel buffer

Tris base	18.15 g
Distilled water	80 ml

Adjust pH to 8.8 with conc. HCl and make the volume upto 100 ml. Store at 4°C.

5. Stacking gel buffer

Tris base	6.05g
Distilled water	80 ml

Adjust pH to 6.8 with con.HCl and finally make the volume to 100 ml with distilled water. Store at 4°C

6. Sample loading buffer (2X)

Tris HCl (1M, pH 6.8)	3.12ml
Glycerol	5 ml
10% SDS	10 ml
2- Mercapto ethanol	1ml
Bromophenol blue	1mg

Adjust the volume to 25 ml using DW.

Mix and store at 4°C

7. Tris Glycine Buffer (5X) (Electrode buffer)

Tris base	15.1g
Glycine	54.0g
DW	900 ml
10% SDS	50 ml

Adjust volume to 1000 ml and pH 8.2.

A working solution of 1X was used.

8. Resolving gel (12%)

Distilled water	6.6ml
30% acrylamide mix	8.0ml
1.5M Tris (pH 8.8)	5.0ml

	10% SDS	0.2ml
	10% APS	0.2ml
	TEMED	0.01ml
9.	**Stacking gel (4%)**	
	Distilled water	3.4 ml
	30% acrylamide mix	0.83 ml
	1M Tris (pH 6.8)	0.63 ml
	10% SDS	0.05 ml
	10% APS	0.05 ml
	TEMED	0.005 ml
10.	**Staining solution**	
	Coomassie Brilliant Blue	1.0 g
	Methanol	250 ml
	Mix by stirring for 30 min. and re-adjust the volume of methanol.	
	Acetic acid	50 ml
	DW	200 ml
	Mix and filter through Whatman filter paper no. 1 and store at amber colored bottle.	
11.	**Destaining solution**	
	Methanol	150ml
	Glacial acetic acid	50 ml
	DW	300 ml

REAGENTS FOR PURIFICATION OF RECOMBINANT PROTEINS

1.	**Lysis buffer (Buffer B) (pH 8.0)**	
	100 mM NaH_2PO4	1.38 g
	10 mM Tris	0.12 g
	8M Urea	48.05 g
	DW	50 ml
	Adjust pH to 8.0 using NaOH, make volume to 100 ml with DW	
2.	**Wash buffer (Buffer C) (pH 6.3)**	
	100 mM NaH_2PO4	1.38 g
	10 mM Tris	0.12 g
	8M Urea	48.05 g
	DW	50 ml
	Adjust pH to 6.3 using HCl, make volume to 100 ml with DW	
3.	**Elution buffer (Buffer E) (pH 4.5)**	
	100 mM NaH_2PO4	1.38 g
	10 mM Tris	0.12 g
	8M Urea	48.05 g
	DW	50 ml
	Adjust pH to 4.5 using HCl, make volume to 100 ml with DW.	

REAGENTS FORWESTERN BLOTTING

1. Phosphate Buffered Saline(PBS)

Sodium Chloride	8 g
Potassium Chloride	0.2 g
Disodium Hydrogen Phosphate (hydrated)	1.15 g
Potassium Dihydrogen phosphate	0.20 g

Add distilled water to make up volume to 1000ml and adjust pH to 7.4.

2. PBS-T

PBS	1000 ml
Tween-20	500 µl

2. Transfer buffer

Tris base	5.82 g
Glycine	2.93 g
Methanol	200ml

Distilled water to make up to 1000ml

5. Blocking buffer

Skimmed milk powder	5 g
TBS-T	100 ml

6. Developing solution

4-chloro-1-napthol	30 mg
Chilled Methanol	10 ml
TBS (pH 7.5)	50 ml
Hydrogen peroxide	60 µl
DAB	6 mg
H_2O_2 (30%)	10 µl
PBS	10 ml

ASSAY FOR DETERMINING THE DNase ACTIVITY

1. TOLUIDINE BLUE-O DNase ASSAY (For 30 ml assay):

Tris (10 mM)	36.34 mg
$MgCl_2$ (10 mM)	28.5 mg
NaCl (100 mM)	175.3 mg
Agar (Difco)	1.5% w/v

pH is set at 7.5

Fish sperm DNA solution (30 mg/ml)	250 µl

It is heated to dissolve completely

Toluidine Blue-O dye (50 mg/ml)	15 µl

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MVSc. (Persuing)	IVRI, Izatnagar, Bareilly, U.P.	2017	8.348/10.0