Studies on Biochemical and Genetic Aspects of the Antimycotic Principle Produced by a Selected Wild type *Enterococcus faecalis*

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by

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Under the supervision of

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CERTIFICATE

This is to certify that the thesis entitled "**Studies on Biochemical and Genetic Aspects of the Antimycotic Principle Produced by a Selected Wild type *Enterococcus faecalis*"** which is submitted for award of Ph.D Degree of the Institute embodies original work done by **Mr. Mohd. Raeesh Shekh**, **ID No. 2008PHXF028G** under my supervision.

Signature in full of the Supervisor: ----------

Name in capital block letters: ----------

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DECLARATION

The material and data contained in this thesis entitled "**Studies on Biochemical and Genetic Aspects of the Antimycotic Principle Produced by a Selected Wild type *Enterococcus faecalis*"** submitted to the Birla Institute of Technology and Science (BITS), Pilani, in partial fulfillment of the requirements for the award of the degree Doctor of Philosophy is my original work and I have not presented any part of this thesis/ work elsewhere for any other degree.

Mohd. Raeesh Shekh

Dedicated to

My Family.....

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Abstract

Aim of this study was to isolate, identify and characterize biochemically and genetically the antimycotic factors produced by *Enterococcus faecalis* and to investigate their safety level and their potential technological properties. Initially 240 bacterial strains from Antarctic and Arctic regions were isolated on Nutrient and Malt, Glucose, Yeast Extract and Peptone (MGYP) agar. As a result of screening, seven bacterial isolates were recovered, all of which inhibited *C. albicans* NCIM 3471.These isolates were further tested against several other strains of *C. albicans*. Three isolates, AGM 111, AGM 108-5 and APR 210, reproducibly exhibited strong antimycotic activity.

Antimicrobial screening revealed *E. faecalis* (APR 210) strain as the potential producer of antimicrobial substance showing good antimicrobial activity in supernatant as well as dialysed concentrate. Biochemically APR 210 strain was Gram positive, non motile, acid producer, catalase negative, oxidase positive, grew in 6.5% NaCl as well as in MRS media, esculin hydrolysis positive, mannitol fermenting, potassium tellurite reducer revealed it to be *E. faecalis*. It showed very good growth from 5°C to 40°C. Two wild type indicator organisms (DI and WI) isolated from hospitals, were also identified up to the species level and found as *C.albicans.* Different indicator organisms like *C.albicans* purchased from Microbial Type Culture Collection (MTCC) Chandigarh and National Collection for Industrial Microorganisms (NCIM) Pune, were resistant against amphotericin B, clotrimazole, miconazole, nystatin and fluconazole antifungal antibiotics. Most of the indicator organisms were sensitive against voriconazole except WI. Investigating the antibiotic sensitivity profile it was determined that most of the clinical isolates of *C.albicans* were multidrug resistant.

The antimicrobial substance retained its biological activity at 90°C for 20 min. and at the pH values of 6.0, 7.0, and 8.0. However activity was reduced by 50% at pH 5.0 and 9.0 and it was partially hydrolyzed by Pronase E and completely by Proteinase K, suggesting its proteinaceous nature and was resistant to trypsin, pepsin, α-amylase and lipase.

The anti-*Candida* protein (ACP) was stable in different organic solvents and surfactants. The storage of ACP at − 80°C for 1 year did not affect the biological activity. Ammonium sulfate salt as well as sodium phosphate buffer did not inhibit ACP activity at the concentration used and did

not modify the result of the assay. The ACP was active against many clinically isolated *C.albicans* (NCIM 3471, NCIM 3129, MTCC 183, MTCC 7315, MTCC 227, MTCC 3958, wild type DI, SC 5314, *P.aeruginosa,* AGM (108-5) and one wild-type *Bacillus* species.

The maximum ACP (1600 AU/ml) was produced extracellularly during middle of stationary phase after 48 h of incubation in modified Tripticase Soya broth (mTSB) at the range of pH 7.4 ± 0.2. Optimization of growth parameters revealed the maximum ACP was produced at pH 7.0 and 8.0 at 15º- 35°C after 48 hrs of incubation with 1.0 % inoculum. The activity was lost after 120 hrs. A moderate level of NaCl enhances the ACP production while increasing.

The ACP was partially purified by ammonium sulfate fractionation, ion exchange chromatography on DEAE Sepharose and gel filtration chromatography using Sephadex G-75. The specific activity was increased after each step having the final purification factor 22.4 and recovery 0.45%. Partially purified ACP had a molecular weight of around ~ 43 kDa in Tricine-PAGE analysis. The ACP activity was also seen onto Tricine gel containing 10% resolving and 5% stacking gel against *C. albicans* MTCC 3958 and MTCC 183.

Safety investigation of ACP was undertaken using haemagglutination and hemolytic activity into account. It was found that the ACP was not able to haemagglutinate human red blood cells up to the concentration of 1.6 mg/ml. At this concentration, the percent hemolytic activity was 3.76 comparatively much less than the hemolytic activities of baciamin and bafilomycin F.

The haemagglutination, hemolytic activity and antibiotics susceptibility of *E. faecalis* showed its non pathogenic characteristics.

The MIC of dialysed concentrate ACP, checked by serial two fold dilution in microtitre plate was found to be 1067 µg/ml against wild type *C. albicans* (DI) whereas against MTCC 183 and MTCC 7315 that was 133 µg/ml. The MIC value of DC against MTCC 3958 was 267 µg/ml.

The N-terminal amino acid sequence of 12 amino acid residues was obtained by Edman degradation. Resulting of that the major sequence DEVYTVKSGDSL and the minor sequence GPGGPGKS'GDS'L was found. The peptide was *de novo* sequenced by ESI-MS, and the deduced combined sequence when compared with other bacteriocins and antimicrobial peptides had no significant sequence similarity. In liquid chromatography electronspray mass spectrometry (LC-ESI-MS/MS), the peptide sequence did not match completely with any protein present in the MASCOT database. However, three significant peptides DIADLQER,

VQAMTTMVK and NQQADAQSQIDALESQVSEINTQAQDLLAK matched with secreted antigen Sag A/B protein produced by *E. faecium*.

Plasmid curing experiment suggests that the antimycotic protein producing gene might be harbored in genomic DNA and was not found induced by mitomycin C.

A genomic library was made in *E. coli* DH5α-pUC 19 host vector system. The transformed host *E. coli* DH5*α* harboring an insert of 1089 bp in pUC 19 showed clear zone of inhibition against *C. albicans* MTCC 3958. The inserts of interest in two positive clones were sequenced. The nucleotide sequence of the clone 40C reveals an open reading frame (ORF) that has maximum similarity with a hypothetical protein pBMB0558_00760 of *Bacillus thuringiensis* CT43 and average homology scores with lipopeptide antibiotic iturin A produced by *Bacillus subtilis.*

These properties taken together might render this antimycotic protein ACP, a potent candidate for treating candidiasis, and its related pharmaceutical application can be established in synergy with other relevant antifungal antibiotics of low dosage.

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List of Abbreviations

E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
C.albicans	*Candida albicans*
MDR	Multidrug resistant
MIC	Minimum inhibitory concentration
AMP	Antimicrobial /antimycotic protein /peptides
ACP	Anti-*Candida* protein
APR	Antarctic penguin rookery
AGM	Antarctic glacial melt
AMPH-B	Amphotericin-B
ATCC	American type culture collection
AMS	Anti-mycotic substance
EDTA	Ethylene di-amine tetra acetate
LAB	Lactic acid bacteria
kDa	Kilo Dalton
NCAOR	National center for arctic and ocean Research
ZMA	Zobell marine agar
MGYP	Malt, glucose, yeast extract and peptone
mTS	Modified tripticase Soya
NCIM	National collections for industrial microorganisms
MTCC	Microbial type culture collection
WI	Wild type isolate

DI	Diabetic isolate
CFS	Cell free supernatant
BOD	Biological oxygen demand
BLAST	Basic local alignment search tool
NB	Nutrient broth
BHI	Brain heart infusion
MR-VP	Methyl red vogues proskauer
AU	Arbitrary unit
CFU	Colony forming unit
BSA	Bovine serum albumin
SDS	Sodium dodecyl sulfate
PMSF	Phenyl methyl sulfonyl fluoride
DTT	Dithio thretol
MWCO	Molecular weight cut off
PAGE	Poly acrylamide gel electrophoresis
TEMED	N, N, N', N' – tetramethylene diamide
APS	Ammonium per sulfate
LC/MS/MS	Liquid chromatography/Mass spectrometry
PVDF	Poly vinyl di fluoride
PCI	Phenol, chloroform and isoamyl alcohol
SDW	Sterile distilled water
RE	Restriction enzyme
PBS	Phosphate buffered saline
CIAP	Calf intestinal alkaline phosphatase

GMWSC	Glacial melt water and sea convergence
FAME	Fatty acid methyl ester
VRE	Vancomycin resistant *Enterococci*
NNISS	National nosocominal infection surveillance system
DC	Dialysed concentrate
PDB	Protein data bank
MALDI-TOF	Matrix assisted laser desorption ionization time of flight
ORF	Open reading frame

CHAPTER 1

INTRODUCTION

Chapter 1 *INTRODUCTION*

1.1. Pathogenicity and Drug Resistance in *Candida albicans*

Candida albicans is a diploid fungus that grows both as yeast and filamentous cells and is a causal agent of opportunistic oral and genital infections in humans (Ryen *et al.* 2004 and Denfert *et al*. 2007). Systemic fungal infections (fungemias) including those by *C. albicans* have emerged as important causes of morbidity and mortality in immunocompromised patients (e.g., AIDS, cancer chemotherapy, organ or bone marrow transplantation). *C. albicans* biofilms may form on the surface of implantable medical devices. In addition, hospital-acquired infections by *C. albicans* have become a cause of major health concerns (Olorode *et al.* 2012). The apparent exaltation of fungal infections may be explained by the increasing number of immunocompromised patients (Lemar *et al.* 2002).

C. albicans is commensal and a constituent of the normal gut flora comprising microorganisms that lives in the human mouth and gastrointestinal tract. *C. albicans* lives in 80% of the human population without causing harmful effects, although overgrowth of the fungus results in candidiasis (Calderone *et al.* 2002). Candidiasis is often observed in immunocompromised individuals such as HIV-infected patients. A common form of candidiasis restricted to the mucosal membranes in mouth or vagina is *thrush*, which is usually easily cured in people who are not immunocompromised. For example, higher prevalence of colonization of *C. albicans* was reported in young individuals with tongue piercing, in comparison to non-tongue-pierced matched individuals (Zadik *et al.* 2010). To infect host tissue, the usual unicellular yeast-like form of *C. albicans* reacts to environmental cues and switches into an invasive, multicellular filamentous form, a phenomenon called dimorphism (Ryen *et al.* 2004).

Having emerged as the major causes of morbidity and mortality in immunocompromised hosts, *Candida* spp., mainly *Candida albicans* and to a smaller extent, also other non-*albicans* species (*C. dubliniensis, C. glabrata, C. guilliermondii, C. krusei, C. lusitaniae, C. parapsilosis, C.*

tropicalis etc.) represent the major group of yeast species recovered from infected individuals (Gozalbo *et al*. 2004). Incidence of *C. albicans* cells acquiring resistance to antifungals has increased considerably, causing serious problems in successful chemotherapy. Both *C. albicans* as well as non-*albicans* species have developed a variety of mechanisms to combat resistance to antifungal drugs (Prasad *et al*. 2005).

Several *Candida* spp., especially *C. albicans,* normally inhabit the oral cavity, respiratory and intestinal tracts, and vaginal cavity of humans and animals (Gozalbo *et al*. 2004). Colonization with *C. albicans* can lead to systemic infection when the host presents risk factors. Predisposing factors for candidiasis (Table 1) include immunosuppressive and cytotoxic therapies, treatment with broad-spectrum antibiotics, AIDS, diabetes, drug abuse, use of catheters and indwelling devices. Depending on the underlying host defects, the microorganism may cause a wide variety of infections ranging from mucosal to life threatening disseminated candidiasis (Gozalbo *et al*. 2004). Therefore, development of candidiasis depends on a delicate balance between the fungi and the host's immune status which determines the commensal or parasitic relationship.

In the recent years, there has been marked increase in the incidence of treatment failures in candidiasis patients receiving long-term antifungal therapy caused by rapidly acquiring multidrug resistance (MDR), which has posed a serious problem in its successful use in chemotherapy (Mishra *et al*. 2007). *C. albicans* pathogenicity depends, in addition to the immune status of the host, on a complex set of microorganism-related putative virulence factors (Naglik *et al.* 2003). These include the secretion of hydrolytic enzymes, antigenic variability, adhesion to host cells and tissues as well as to inert substrates, dimorphic transition (yeast to hypha), phenotypic switching or the ability to switch among different cell phenotypes and modulation of the host's immune response (Cutler 1991; Calderone 2002; Chaffin *et al*. 1998; Martinez *et al*. 1998; Soll 1992 and Naglik *et al*. 2003).

Expression of most virulence factors is regulated *in vitro* by environmental parameters and *in vivo* it depends on the stage of infection and varies also according to host species or tissues. It is generally accepted that dimorphism (budding yeast or filamentous form or hypha) is virulence

Table1. Abnormalities caused by *C.albicans*, locations, symptoms and predisposing factors (Mishra *et al*. 2007).

Type	Location	Symptoms	Predisposing factors
Superficial			
Oral thrush	Oropharynx	White lesions resembling milk curd on the surface of throat, tongue, and gum linings	Old age, infancy
Stomatitis	Palate	Erythema,oedema of palate	Old age
Leukoplakia	Inner cheek surface	Chronic lesions	Tobacco smoking
Vulvo vaginal	Vagina,perinal area	White discharge,intense arythema	Pregnancy,diabetes
Candida onychea	Brown greenish discoloration of nails	Swollen,painful inflammation of nail	Occupational hazards
Systemic			
Oesophagial	Oesophagus	Dysphagia,retrosternal pain	Fatally ill patients,AIDS
Gastric	Stomach	Lesions,vomiting	Preexisting lesions
Candidiasis of lower respiratory tract	Bronchi,lungs	Lesions	Aspiration
Candiduria	Kidney,urinary tract	Microabscesses in urinary tract	Diabetes,old age drug abuse
Candida endocarditis	Heart,aortic and mitral valve	Intracardiac vegetation blocking blood vessels	Cardiac pulmonary bypass,open heart surgery
Candida meningitis	Granulomas of ventriclelesions	microabscesses, headaches	No patricular factors
Candida arthritis	Necrosis of cartilages,abscess formation	Swelling,painfull joints	Intraarticular steroid injection,trouma,cancer patients
Disseminated			
	Eye,skin,blood		Leukimia,burn patients

trait, and is co-regulated with other virulence factors associated with the cellular morphology (Brown *et al*.1999 and Ernst *et al*.2000).

In addition to the yeast hypha transition, *C. albicans* may undergo also another type of morphological change called phenotypic switching, involving the spontaneous and reversible generation of different morphological and physiological states expressing different patterns of pathogenicity-related traits (virulence factors, antigenicity and resistance to antifungal drugs) readily observed by the morphology of the colonies (Soll *et al*. 1992 and 1997). Both processes, dimorphic transition and switching, confer on *C. albicans* the ability to generate variants allowing a better selective adaptation to changing environmental conditions, and particularly to evade the host's immune system. Gastrointestinal tract and oral cavity are observed to be colonised by *C. albicans* (Calderone *et al*. 2002). The key to its pathogenicity is the ability to colonise available niches within the host facilitated by phenotypic switching between morphogenic forms. When conditions are favourable they can invade the oral and vaginal epithelium and in severe cases can invade the blood stream and disseminate, leading to a systemic infection (Calderone *et al*. 2002).

To combat candidiasis several classes of drugs have been developed that act on the cell membrane or cell wall to exert their antifungal action (Denning *et al*. 2003; Chen *et al*. 2005 and Kleinberg *et al*. 2006). As *C. albicans* can survive in a variety of niches within the host it has a series of well characterised stress responses that are activated during changes in temperature, pH and osmolarity (Cannon *et al*. 2007). These stress responses contribute to resistance from host defence mechanisms. Resistance is frequently due to genetic mutation targeted by these drugs or enzymes involved in metabolic pathways. For example, the cell wall integrity pathway is responsible for glucan synthesis and cell wall repair (Navarro-Garcia *et al*. 1998). A change in this pathway can lead to echinocandin resistance. Resistant strains have mutations in the 1, 3-β glucan synthase subunits Fks1p and Gsc1p (Balashov *et al*. 2006 and Baixench *et al*. 2007). However, resistance to the echinocandins has been reported only rarely, this could be due to their relatively recent introduction (Perlin *et al*. 2007). The increase in minimum inhibitory concentration (MIC) associated with a decrease in susceptibility is greater with caspofungin and micafungin than anidulafungin. The reason for these differences is not yet fully understood. *C. albicans* strains that show azole resistance may display mutations in ERG3, lowering the ergosterol content in the membrane and so reducing the effectiveness of both the azoles and

amphotericin B (Sanglard *et al*. 2003). *Erg11p* is the target of azole drugs and point mutations in this gene reduce drug affinity leading to resistance (White *et al*. 1998). The regulators of *ERG* genes have also been implicated in resistance, for example, deletion of *UPC2* resulted in *C. albicans* cells that were hypersensitive to fluconazole and ketoconazole, whereas overexpression increased resistance to these drugs (MacPherson *et al*. 2005). Clinical strains of *C. albicans* also overexpress *Cdr1p* and *Cdr2p*, two phospholipid transporters that can enhance azole resistance, and deletion of these genes decreases azole action by 8-fold (Tsao *et al*. 2009). It has also been reported that inactivation of a sterol (Δ5, 6-desaturase) involved in the final stage of ergosterol synthesis decreases azole susceptibility (White *et al*. 2007).

The drug resistance of *C. albicans* to amphotericin B is rare and not fully understood. A decrease in ergosterol content decreases susceptibility and is thought to be associated with mutations in the genes involved with the regulation of ergosterol production (Ghannoum and Rice 1999). The susceptibility of *C. albicans* biofilms has also been investigated and it was found that several genes encoding enzymes of the ergosterol (*ERG1, ERG2*) and β-1, 6-glucan (SKN1, KRE1) pathways were differentially regulated. It was hypothesised that changes to susceptibility originate from changes to both the cell membrane and cell wall (Khot *et al*. 2006).Fungal infections are widely treated with triazole antifungal agents, such as fluconazole. Unfortunatly, long term therapies have led to the emergence of fluconazole resistant *C.albicans* strains that are cross resistant not only to the other azoles but also to amphotericin B (Kelly *et al*. 1996). This obviously points to a pressing need for new antifungal agents, e.g., antimicrobial proteins or peptides (Hancock and Chappel 1999, Hancock 1999).

1.2. Microbial Solutions to Microbial Problems

Microbial solutions to the microbial problems are one of the best possible solutions to these emerging problems. The antimicrobial peptides naturally produced from extremophilic organisms can invade MDR problems (Das *et al*. 2006, Hop *et al*. 2011 and Okazaki *et al*. 2006).These compounds vary in spectrum and mode of activity, molecular structure and

molecular mass, thermostability, pH range of activity, and genetic determinants (De Vuyst *et al*. 1994; Nes *et al*. 1996; Nissen- Meyer and Nes *et al*. 1997; Moll *et al*. 1999; Ennahar *et al.* 2000; Cleveland *et al*. 2001; McAuliffe *et al*. 2001; Riley *et al*. 2002 and Eijsink *et al*. 2002).

Psychrophilic microorganisms are widely prevalent on earth, as there is a vast area where temperatures constantly remain below 10°C, such as in deep-sea waters, mountains and Polar regions. The constraints on and sustainability of life in frozen environments are of considerable importance in a number of contexts, from polar microbial ecology and astrobiology to cryopreservation and other industrial applications (Rothschild and Mancinelli 2001; Singh and Elster 2007). Psychrophilic bacterial strains, and particularly their enzymes, that are able to perform catalysis efficiently at low temperatures have been proposed for use in a number of biotechnology applications (Priscu *et al*. 1999 and Groudieva *et al*. 2004). In view of the severe environmental conditions prevailing in Antarctica and the Arctic, it has been argued that the production of extracellular antimicrobial compounds would be a particular advantage in reducing interspecies competition (O'Brien *et al*. 2004). The producer strains amonst the microorganisms in those microhabitats exhibit antimicrobial prowess to maintain community structure of the ecological niches. Therefore, it was suggested that Polar regions may be viewed as a vast untapped reservoir of microorganisms with manifold antibiotic potential (O'Brien *et al*. 2004 and Shekh *et al.* 2011).

1.3. Antimicrobial Peptides (AMPs)

Antimicrobial and antimycotic peptides are small cationic and amphipathic molecules, generally with fewer than 50 amino acids. These peptides are omnipresent and have been isolated from prokaryotes and eukaryotes in the plant, bacterial, fungal, and animal kingdoms (Hoffmann 1999 and Bulet *et al*. 2004). Nature has strategically placed antimicrobial and antifungal peptides as first line of defenses between the host organism and its surrounding environment, because these peptides are able to inhibit quickly a wide spectrum of infectious microbes without significant toxicity to the host organism (Otvos *et al*. 2000).

Although antimicrobial peptides (AMP) are the primary means of combating organisms in lower forms of life, these peptides have an adjunct role to the immune system in phylogenetically more advanced organisms. Indeed, cationic peptides in humans have an important role and they are produced and secreted by several different tissues, including salivary glands, skin, eye, liver, as well as epithelial and platelet cells and neutrophils (Ganz *et al.* 1997). Several antifungal peptides display selective toxicity for the microbial target by identifying conserved molecular determinants of pathogens (Thevissen *et al.* 1999 and 2000). A classic example is the echinocandin family which targets 1, 3 β glucan synthase, an enzyme essential for cell wall integrity of fungi (Schmatz *et al*. 1992).

In most instances, however, AMPs are less specific in their targeting and this result in their exhibiting a broad spectrum of inhibitory/cidal activity not only against fungi but also against bacteria and envelope-containing viruses (Jigami *et al*. 1999). Broad spectrum AMPs often target and lyse the membranes of the microbes, yet these peptides frequently have less proclivity to lyse mammalian cell membranes such as those of red blood cells. The interaction between AMP and target microbes is complex, but the positive charge of the peptides is essential to its binding with negatively charged membrane/wall elements such as the mannoproteins in yeasts (Jigami *et al*. 1999). Moreover, despite targeting and lysing microbial membranes, the potencies and spectra of activities of these broad spectrum AMPs against different classes of microbes vary and depend on the membrane composition of the pathogen and the structure of the peptide. Much remains to be learnt about the subtle differences in microbial membranes that may affect efficacy of the AMPs (Matejuk *et al*. 2010).

Diversity of antimycotic peptides

Antimycotic peptides are found among all classes of life, from prokaryotes to eukaryotes. AMPs are much more common in higher class of life Figure 1, (Lucca *et al*. 1999).

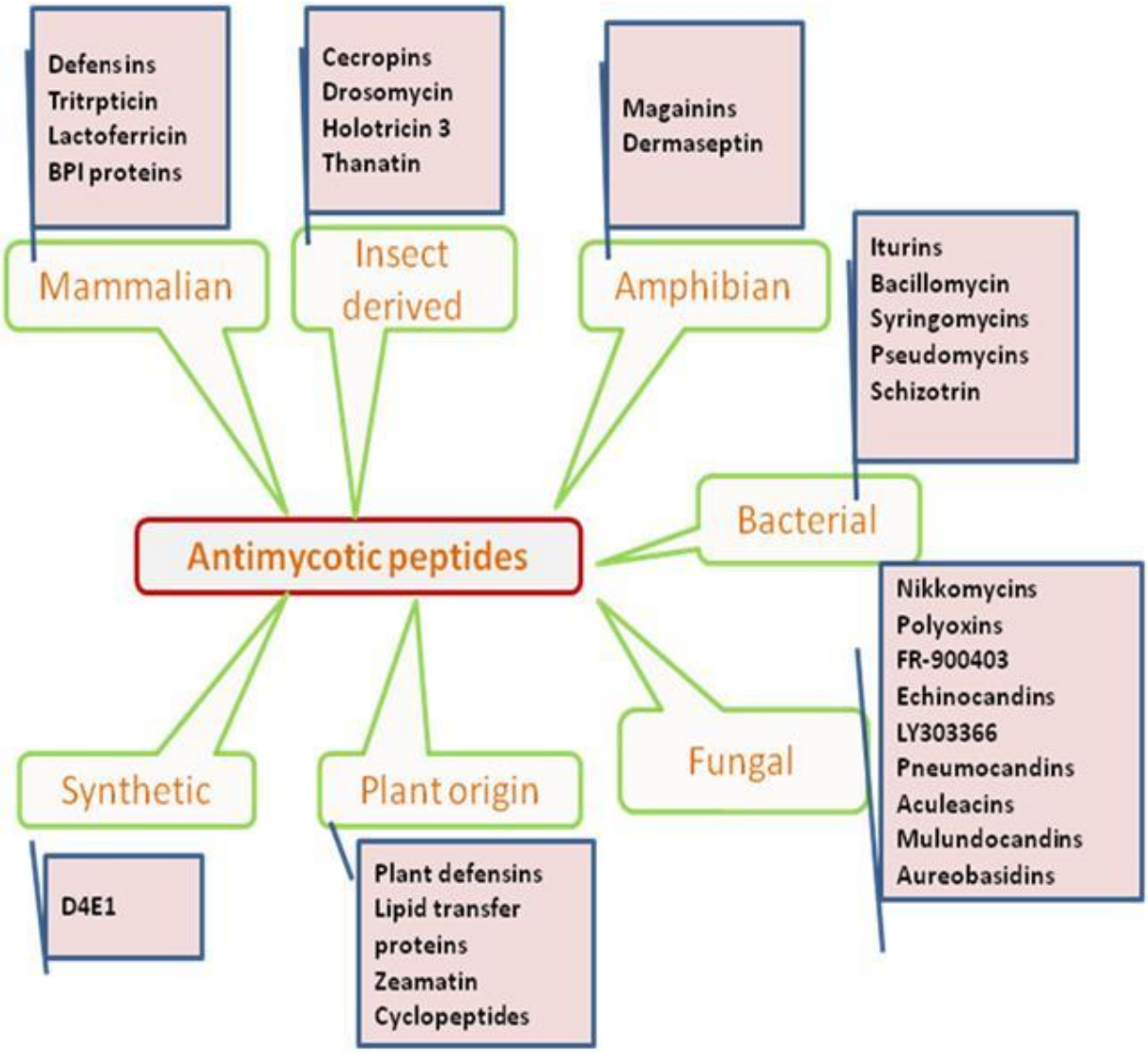


Figure1. Diversity of antimycotic peptides/proteins from higher and lower class of life.

1.4. Bacteriocins from *Enterococcus* species

Since 1955, when the first bacteriocin-like substance within the group D streptococci was reported by (Kjems, 1955), a large number of enterocins has been studied. Kramer and Brandis, 1975, reported the anti-Listeria activity of the enterocin E1A produced by *E. faecium* E1. The

first enterocin purified to homogeneity was the enterocin AS-48 produced by *E. faecalis* S-48 (Galvez *et al*. 1989 and Martinez-Bueno *et al*. 1994), which was defined as a cyclic peptide antibiotic. However, many of these molecules have not been purified to homogeneity. *Enterocins* are found within the class I, class IIa, class IIc, and class III bacteriocins.

The bacteriocins produced by the *E. faecalis* strains show a narrow spectrum of activity, mainly against other *Enterococcus* spp. compared with those from the *E. faecium* strains showing a broader spectrum of activity, against indicator strains of *Enterococcus spp*., *Listeria spp., Clostridium spp.* and *Propionibacterium spp.* Enterococcal bacteriocins characterized at the biochemical level (enterocins A, B, I, and P), proved to be strong inhibitors of food borne pathogens such as *L. monocytogenes*, since they all belong to the class II of LAB bacteriocins (Aymerich *et al.* 1996;Casaus *et al.* 1997;Cintas *et al*. 1997 and Floriano *et al*. 1998). These strains may be of great technological importance in cheese manufacture when used as starter and/or coculture, since *L. monocytogenes* has been shown to be able to survive the manufacture and ripening conditions of cheeses. Aymerich *et al*. 2000 experimented and recommended the application of enterocins as bio preservative against *L. innocua* in meat products.
As the literature on antifungal proteins from bacteria is rather scanty compared with that on bacteriocins, therefore, there is a pressing need to explore and isolate bacteria from new and unexplored sources capable of producing novel AMP and to characterize them for further applications.

1.5. *Enterococci* and Health Perspective

Enterococcus is a genus of lactic acid bacteria of the phylum Firmicutes. Members of this genus were classified as Group D *Streptococcus* until 1984 when genomic DNA analysis indicated that a separate genus classification was appropriate (Schleifer and Kilpper–bazz, 1984). The genus *Enterococcus* is comprised of Gram-positive, microaerophilic cocci, which are not motile and occur in chains or pairs. These are difficult to distinguish from *Streptococci* on physical characteristics alone. The genus is defined by a combination of antigenic, haemolytic, and physiological characteristics. *Enterococci* are facultative anaerobic organisms (Fischetti *et al*.

2000). They typically exhibit gamma-hemolysis on sheep's blood agar (Schleifer *et al*.1984). Two species are common commensal organisms in the intestines of humans, *E. faecalis* (90-95%) and *E. faecium* (5-10%), Ryan and Ray (2004) *E. faecalis* is resistant to many commonly used antimicrobial agents (aminoglycosides, aztreonam, cephalosporins, clindamycin, penicillins, nafcillin, oxacillin and trimethoprim-sulfamethoxazole). Exposure to cephalosporins is a particularly important risk factor for colonization and infection with enterococci. Resistance towards vancomycin and ampicillin has increased alarmingly over the past few years. In 1997, 52% and 83% of isolates sampled in the United States were vancomycin and ampicillin resistant, respectively (Ryan and Ray, 2004).

1.6. Gaps in Existing Research

The past decade has witnessed a dramatic growth in knowledge of natural peptides. The emergence of fungal pathogens resistant to current therapies further compounds the dearth of antifungal agents. Currently available antifungal compounds act on targets also found in mammalian cells (Anthony *et al*. 1999), which may result in toxicity or an adverse drug interaction. It is therefore imperative to find antifungal compounds that are not toxic to mammalian cells.

Although the azole antifungal agents are considered to be less toxic than amphotericin-B, their efficacies against deep-seated, life-threatening mycoses are not satisfactory. In addition, it has been reported that the frequency of isolation of multiazole-resistant strains of *Candida* species other than *C. albicans* is increasing (Hitchcock *et al*. 1993). Therefore, there is a critical need for new antifungal agents which are strongly candidacidal and have a broad spectrum of activity and have fewer side effects (Tawara *et al*. 2000 and Philips *et al*.2003).

The growing problems of resistance to conventional antibiotics and the need for new antibiotics stimulated resurgent interest in the development of antimicrobial peptides as human therapeutics as was envisaged by Zasloff (2002). Most of the antimicrobials developed or discovered have been rejected or found even less effective in animal models only at very high doses, often very

close to the toxic doses of the peptide, reflecting an unacceptable margin of safety. However, considering that the emergence of resistance against antimicrobial peptide is less probable that observed for conventional antibiotics and provides the impetus to develop antimicrobial peptides into the therapeutically useful agents (Zasloff 2002). Therefore, a continuous need for new products and methods for developing new anti-microbial agents exist and all these essential information warrant us to isolate new organism from different sources like sea and extreme environments (O'Brien *et al*. 2004, Lindermuth *et al*. 2001 and Walsh *et al.* 2003).

In an earlier report pentocin TV35b, a bacteriocin-like peptide produced by the *Lactobacillus pentosus* (isolated from fornix secretions of the vagina) inhibited *C. albicans* MCB1, MCB2, MCB 3 and 4. However the activity was not at all very strong as shown by the zone of inhibition which was only 10 -15 mm (Okkers *et al.*1999). Later working on the same line the antimicrobial substance termed as a "class II lactic acid bacterium bacteriocin" produced by *Lactobacillus rhamnosus* L60 a human vaginal isolate was reported to inhibit *C. albicans, K. pneumoniae* and *E. coli* (Pascual *et al*. 2008). Recently *Campylobacter* strains were reported to inhibit *C. albicans* ATCC 44859 (Workman *et al*. 2008) and the zone of inhibition was merely 8 mm in diameter.

The current investigation therefore is aimed to tackle the drug-resistant harmful fungal pathogens with the help of antimicrobial peptides (AMP) alone and or in suitable synergy with antibiotics and the outcome may show the path leading to prophylactic and therapeutic alternative. Products and methods responsive to this need would ideally involve substantially non-toxic compounds available in large quantities. Ideal compounds would have a rapid effect and a broad spectrum of fungicidal or fungistatic as well as bactericidal or bacteriostatic activity against a variety of different microbial species. Even if an antifungal agent is not effective on its own, when administered in conjunction with other antifungal or antibacterial agents, the combination may be more effective or the combination may permit a reduction in the amount of the additional anti-fungal or antibacterial agent.

1.7. Aims and Objectives

Aim of this study was to isolate, identify the antimicrobial peptide (AMP) producing strains from extremophilic environment and characterize antimicrobial peptide or protein of interest.

Following were the specific objectives of present research work.

1. To identify the most promising wild-type extremophilic bacterial isolate having a strong anti-mycotic activity against different multi-drug resistant *C. albicans* (that is the identification of the selected isolate up to species level by polyphasic analysis).

2. To study the genetic factors associated with the production of the anti-mycotic substance (AMS).

3. To characterize biochemically the AMS and to sequence the purified antimycotic substance by N-terminal amino acid sequencing followed by cloning and expression.

CHAPTER 2

REVIEW OF LITERATURE

Chapter 2 *REVIEW OF LITERATURE*

2.1. Antimicrobial Peptides / Proteins (AMPs)

Antimicrobial peptides are evolutionarily ancient weapons. Their widespread distribution throughout the animal and plant kingdoms suggests that antimicrobial peptides have served a fundamental role in the successful evolution of complex multicellular organisms (Zasloff *et al*. 2002).

Antimicrobial peptides are common in archea, eubacteria, protests, plants and invertebrates, revealing that these peptides were present early in evolution (Lehrer *et al*. 2004). Antimicrobial peptides have been also found in all class of eukaryotes (Boman *et al*.1995) and designated as small polypeptides less than fifty amino acid residues, with the ability to directly kill bacteria, fungi and enveloped viruses. The activity spectrum is unique for every peptide and a single amino acid substitution can affect the activity (Aley *et al*. 1994 and Kragol *et al*. 2001).

Antimicrobial peptides, including both cationic and neutral peptides, are secreted from both Gram-positive and Gram-negative bacteria (Hancock *et al*. 1999). These have been classified within the bacteriocins which also include proteins (Abee 1995; Baba 1998; Jack and Jung 1998). Bacteriocins are generally able to kill specific bacterial competitors while causing little or no harm to the host bacterium, due to posttranscriptional modification and/or specific immunity mechanisms (Baba 1998). Some peptide bacteriocins, including the *Escherichia coli* 7-amino-acid peptide microcin C7, which inhibits protein synthesis, and the *Lactococcus* peptide mersacidin, which inhibits peptidoglycan biosynthesis, have specific mechanisms which inhibit bacterial functions. However, most of these peptides, e.g., nisin and epidermidin, are thought to permeabilize target cell membranes (Baba 1998 and Wieprecht *et al*. 1997).

Very less antimicrobial peptides were discovered till date from bacteria, and various strains of *Bacillus subtilis* were reported to produce the iturin peptide (Lucca *et al*.1999). They are small cyclic peptidolipids characterized by a lipid-soluble β-amino acid linked to a peptide containing

D and L amino acids (Peypoux *et al*. 1973). Iturins affected membrane surface tension, which caused pore formation and which resulted in the leakage of K^+ and other vital ions, paralleling cell death (Besson *et al.*1984; Lawyer *et al*. 1996 and Thimon *et al*.1992). One family member, bacillomycin F, inhibited the growth of fungi including *Aspergillus niger*, *C. albicans*, and *Fusarium oxysporum* (Landy *et al*. 1948 and Mhammedi *et al*. 1982). Unfortunately, bacillomycin L and iturin A have been found to be hemolytic, which may reduce their potential use as antifungal drugs (Latoud *et al*. 1986).

Members of the *Pseudomonas syringae group* produce small cyclic lipodepsipeptides known as syringomycins (Segre *et al*. 1989). Sorenson *et al*. (1996) published a thorough study of the potent fungicidal properties of several compounds produced by *P. syringae*, including SE, syringotoxin B, and syringostantin A. These compounds were fungicidal for *Candida*, *Cryptococcus*, and *Aspergillus* isolates. A 12% (w/v) ointment of syringomycin was effective in controlling vaginal candidiasis in a murine model (Sorensen *et al*. 1998). *P. syringae* also produced the pseudomycins, another family of peptides with broad-spectrum antifungal activity (Harrison *et al*. 1991).

Polyoxins, which are produced by *Streptomyces cacaoi*, were active against isolated chitin synthases but had variable activity against intact organisms (Hori *et al*. 1974 and Suzuki *et al*. 1965). Polyoxin D was found fungistatic for *C. albicans* at concentrations of 500 to 2,000 mg/ ml, depending on the strain, and inhibited *C. neoformans* growth (Becker *et al*. 1983). Notably, polyoxin D reduced the ability of *C. albicans* to bind to buccal epithelial cells by as much as 58% compared to the binding ability of controls (Gottlieb *et al*. 1991).

Moyne *et al*. (2001) purified an antifungal peptide that shown the antifungal activity against *A. flavus*. Two lipopeptides were purified with anion exchange and gel filtration chromatography. Their masses were determined to be 1045 and 1059 m/z with mass spectrometry, and their peptide moiety was identical to bacillomycin D. A few years later, a honey-isolate *B. subtilis* H215 showed strong antifungal activity against *B. fulva* H25 (Lee e*t al*. 2008). The antifungal peptide was purified by 20% ammonium sulfate precipitation of the bacterial culture supernatant,

followed by Octyl-Sepharose CL-4B and reverse phase-high performance liquid chromatography. The five active fractions were lyophilized and subjected to mass, tandem mass spectrometry and amino acid analysis to deduce their corresponding molecular masses and structural characteristics. The five peaks were determined to be identical to bacillomycin F, varying in the length of the fatty acid chain moiety from C14 to C16. (Lee *et al*. 2008).

An antifungal protein designated as baciamin (Wong *et al*. 2008) was purified and exhibited a molecular mass around 50 kDa. Baciamin manifested a broad-spectrum of antifungal activity and could induce membrane permeabilization of tested fungi. Its antifungal activity was retained after incubation with trypsin and EDTA. Various ions tested did not affect its antifungal activity. Separately an anteiso-C17 isoform (Fickers *et al*. 2009) of the lipopeptide mycosubtilin produced by a genetically engineered *B. subtilis* strain was reported and demonstrated a high-level production (880 mg/l). Antifungal activity of this isoform, as determined via culture, fluorometric and cell leakage assays, suggest its potential therapeutic use as an antifungal agent, in particular on *Candida* sp (Fickers *et al*. 2009).

Very recently Tabbene *et al*. (2011) reported that *B. subtilis* B38, isolated from soil, showed antimicrobial activity against human pathogenic *C. albicans* species. In his study anti-Candida compounds designated a1, a2 and a3 were purified from culture supernatant and identified using matrix-assisted laser desorption/ionization time-of-flight MS as analogues of bacillomycin D-like lipopeptides. The compound a3 displayed the strongest fungicidal activity against pathogenic *C. albicans* strains. It was even more active than amphotericin B with a lethal concentration of 59.07 vs. 135.26 mM of the antimycotic drug against the pathogenic strain *C. albicans* spp. 311 isolated from finger nails. Only moderate or weak anti-*Candida* activity was recorded for a1 and a2 compounds. Furthermore, a3 showed the highest hemolytic activity, reaching 50% hemolysis at 22.14 mM, whereas a1 and a2 displayed a limited hemolysis at 68.26 and 37.41 mM, respectively. His findings suggest that the acyl chain length of bacillomycin D-like lipopeptides play a major role in hemolytic and antifungal activities (Tabbene *et al*. 2011).

2.1.1. Classification

Since the structures of antifungal peptides are disparate and the incomplete knowledge of their mechanisms of action, classification of the various antifungal peptides is a daunting task (Matejuk *et al*. 2010). Whereas some lipopeptides (e.g., echinocandins) or histidine-rich (e.g., the linear histatins or branched HK) peptides have primarily antifungal activity, membrane-disrupting peptides (e.g. Magainins, protegrins) inhibit a diverse group of microorganisms including fungi, bacteria, and viruses. Structurally, linear cationic antifungal peptides (e.g., LL-37, magainins) form α-helical structures in a hydrophobic milieu while cysteine-containing peptides containing from one to multiple disulfide bonds (e.g., protegrins and defensins) form β-sheet enriched structures (Matejuk *et al*. 2010). The formation of these α–helical and/or β sheet secondary structures may increase the amphipathicity of the peptides and enable them to act specifically with their targets in the fungal membrane. In addition to histidine-rich peptides such as histatins, other peptides (e.g., apidaecins, indolicidin) have a high percentage of certain amino acids such as proline and tryptophan. Interestingly, potent antifungal linear peptide fragments from larger proteins (e.g., lactoferrin and lysozyme) are able to inhibit fungi because of multiple direct and indirect effects (Lopez-Exposito *et al*. 2008). Nearly 1200 antimicrobial peptides have now been identified (Matejuk *et al*. 2010).

2.1.2. Antimycotic Peptides from Lactic Acid Bacteria

Lactic acid bacteria (LAB) constitute a group of bacteria that have morphological, metabolic and physiological similarities, and they are also relatively closely related phylogenetically. The general description of the bacteria within the group is Gram positive, non-sporulating, non-respiring cocci or rods, which do, through fermentation of carbohydrates, produce lactic acid as their major end product. The common agreement is that there is a core group consisting of four genera; *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* (Axelsson *et al*. 1998). Recent taxonomic revisions have proposed several new genera and the remaining group now comprises the following: *Aerococcus, Alloiococcus Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactococcus, Oenococcus, Tetragenococcus, Vagococcus,* and

Weissella. Lactobacilli, carnobacteria and some *weissella* are rods while the remaining genera are cocci (Axelsson *et al*. 1998).

Ribosomally synthesised antimicrobial peptides are found in a broad range of organisms, such as mammals, birds, amphibians, insects, plants and microorganisms. Although the group of antimicrobial peptides is diverse, they generally share some features; a hydrophobic and a hydrophilic end, a size of 20- 50 amino acids, and cationic properties (Nissen-Meyer *et al*. 1997 and Hildeng-Hauge *et al*. 1998). LAB produce antibacterial, ribosomally synthesised, peptides, generally termed bacteriocins (Nes *et al*. 1996).

A large number of bacteriocins have been characterized from lactic acid bacteria in recent years. The bacteriocins from lactic acid bacteria are commonly divided into three groups: class I – the lantibiotics; class II – the heat stable unmodified bacteriocins; class III the larger heat stable bacteriocins (Nes *et al*. 1996 and Nes *et al*. 2000). These compounds are generally only active against closely related bacterial species and there is no evidence that bacteriocins have any effect on growth of yeast or moulds (Nes *et al*. 1996 and Nes *et al*. 2000).

There are only few reports on the production on antifungal peptides produced by lactic acid bacteria. Several authors have reported that the antifungal activity of LAB is lost after treatment with proteolytic enzymes. Batish *et al.* (1989) claimed that the antifungal substance produced by a lactic acid bacterium was of proteinaceous nature since it was degraded by proteinases. However, they did not present any results supporting these claims and did not characterise the active compound in any detail. Roy *et al*. (1996) isolated a *L. lactis* subsp. *lactis* CHD 28.3 with antagonistic activity against several filamentous fungi. After enzymatic treatment with chymotrypsin, trypsin and pronase E, the antifungal activity disappeared, indicating a proteinaceous nature of the antifungal substance. Gourama *et al*. (1997) found that the inhibitory effect of a *L. casei* strain against two *Penicillium* species was slightly reduced by treatment with trypsin and pepsin, but the compound was not characterized further. Gourama *et al*. (1995) and Bullerman *et al*. (1997) showed that a commercially available silage inoculant with a

combination of *Lactobacillus* species (*L. plantarum, L. bulgaricus* and *L. acidophilus*) exerted antifungal and anti-aflatoxin activity against *A. flavus*.

2.1.3. Characterization, Applications and Health Related Issue of Some other Antimycotic Peptides

Peptides with primarily antifungal properties

The potential advantage offered by the less ubiquitous antifungal peptide is that the therapeutic window for peptides specific for fungi is greater than that of peptides with broad antimicrobial activity (Matejuk *et al.* 2010). In contrast to broad-spectrum antimicrobial peptides, which induce membrane lysis, most of these antifungal peptides have specific targets that are intracellular, on the cell membrane, or on the cell wall. Because of the diverse targets, the structure of these AMPs can vary significantly and include linear, open ended cysteine-rich cyclic peptides, and cyclic lipopeptides (Matejuk *et al*. 2010). Nevertheless there are many peptides, either derived from natural sources or synthetically prepared, exhibit primarily antifungal activity.

1, 3-β-Glucan synthesis inhibitors

These antifungal peptides are cyclic lipoproteins that noncompetively inhibit the multiunit membrane-integrated enzyme, β-glucan synthase, critical for cell wall integrity. Inhibition of β-glucan synthase results in destabilizing the cell wall, leading to susceptibility to osmotic stresses and cell lysis (Matejuk *et al.* 2010; Georgopapadakou *et al.* 1996 and Turner *et al.* 1997). In addition to the cell wall, 1, 3- β-glucans have a role in the division septum and assembly of the acropore wall; consequently, these structures are also sensitive to the synthase inhibitors. β-glucan synthase has a widespread distribution in fungi including *Candida*, *Aspergillus*, *Cryptococcus*, and *Pneumocystis* species. This family includes the echinocandins, pneumocandins, mulundocandins, aculeacins, and WF11899 (Georgopapadakou *et al.* 1996 and Turner *et al.* 1997).

Echinocandins and Pneumocandins: Several analogs from these two classes of β- glucan synthase inhibitors have shown promise in preclinical or clinical studies for treatment of invasive and systemic *Candida* and *Aspergillus* infections (Benz *et al.* 1974). Of the three subfamilies of echinocandins (B, C and D), analogs of group B have been most useful in the development of antifungal drugs. Echinocandin B, produced by *A. nidulans* and *A. rugulosus*, was found to have potent antifungal activity with an MIC between 0.20 and 0.35μg/ ml for *Candida* spp. (Benz *et al*. 1974).

Aculeacins (A-D, F), isolated from *Aspergillus aculeatus*, have potent antifungal activity (Mizuno *et al.* 1977, Satoi *et al.* 1977 and Mizoguchi *et al*. 1977). In general, aculeacins have a less MIC 0.31 μg/ ml for most *Candida* spp., but they are not active against *C. tropicalis* or most filamentous fungi (Iwata *et al*. 1982). When aculeacin A was compared with pneumocandin A_0, the aculeacin induced significantly more hemolysis at a lower concentration and was slightly less effective *in vivo* against systemic *C. albicans* infection in a mouse model (Fromtling *et al*. 1989).

Mulundocandins are produced by *Aspergillus syndowi* var. *mulundenis* (Mukhopadhyay *et al*. 1987 and Roy *et al*. 1987) and are effective against *C. albican*s (MIC, 0.5–4.0 μg/ ml), *C. glabrata* (MIC, 2.0 to 4.0 μg/ ml), and *C. tropicalis* (MIC, 1.0–8.0 μg/ ml). Against other species of *Candida*, mulundocandins are less active and show little to no activity against *C. neoformans* or filamentous fungi (Hawser *et al.* 1999).

WF11899A: WF11899 A, B and C demonstrated potent anti-*Candida* activities that are superior to cilofungin, and equivalent to fluconazole; unfortunately, they lyse mouse red blood cells at low concentrations (Iwamoto *et al*. 1994).

Inhibitors of Chitin in the Cell Wall

Nikkomycins, produced by *Streptomyces tendae* and *S. ansochromogenes*, and *polyoxins*, produced by *S. cacaoi*, are the most widely studied peptidyl nucleoside inhibitors of chitin synthase (Hori *et al.* 1974, Brillinger *et al.* 1979 and Chen *et al*. 2000).

Nikkomycins are able to inhibit chitin synthesis in *C. albicans* both *in vitro* and *in vivo* studies (McCarthy *et al*. 1985) and it is not toxic to human cells and showed significant activity against *Coccidiodes immitis*. *Blastomyces dermatitidis* and moderate activity against *Hisotoplasma capsulatum* (Hector *et al*. 1990 and Clemons *et al*. 1997), but these agents are not active against filamentous fungi. These inhibitors have limitations because of their unfavorable pharmacokinetics.

Aureobasidins: There are 18 members of the Aureobasidins family, produced by *Aureobasidium pullulans*. They are cyclic depsipeptide lipophilic antibiotics made up of eight amino acids and an α-hydroxyacid (Ikai *et al*. 1991). Two modes of action have been proposed for aureobasidins: one is based on disruption of cell wall/membranes by altering the assembly of actin and chitin (Endo *et al*. 1997) and the other is based on interrupted synthesis of sphingolipids (Nagiec *et al*. 1997).Several members of the Aureobasidin family are active against *Candida* spp. (e.g., MIC, Aureobasidin A, <0.05 to 0.2 μg/ ml), and *C. neoformans* (e.g., MIC, Aureobasidin A, 0.78 μg/ ml) (Takesako *et al*. 1993).

Membrane-active Selective AMPs

Rs-ARF2, isolated from radish seeds, is a 50 amino-acid residue plant defensin that has an α helix and three-stranded β-sheets stabilized by four disulfide bridges. Rs-ARF2 shares structural and functional homology with other plant defensins, HsAFP1 and DmAMP1, and the insect defensin, heliomicin. RsAFP2 (10 μM) inhibited *C. albicans* and *C. krusei* by 99.8% and 91.1%, respectively. *C. glabrata*, which does not contain this fungus-specific ceramide, was not inhibited. Furthermore, the MICs of RsAFP2 toward *A. flavus* and *Fusarium solani* were 0.7 and 0.04 μM, respectively (Thevissen *et al*. 2007).

Iturins, produced by *B. subtilis*, are cyclic peptides with a lipid-soluble β-amino acid linked to an array of D and L amino acids. Iturins act on microbial membranes causing pore formation and leakage of key ions of fungi (Besson *et al*. 1984), but their antimicrobial activity is limited primarily to fungi, with little effect on bacteria. Unfortunately, iturins are toxic to mammalian

cell membranes. In contrast to most other antimicrobial peptides which are cationic, iturins may be anionic (bafilomycin L) or neutral (iturin A). One member of the family, bafilomycin F effectively inhibits *A. niger* (MIC, 40 μg/ml), *C. albicans* (MIC, 40 μg/ml), *C. tropicalis* (MIC, 40 μg/ml), and several phytopathogens such as *Mycosphaerella pinodes* (MIC 10 μg/ml) (Mhammedi *et al*. 1982). In contrast to its potent antifungal activity, bafilomycin F modestly inhibits the bacterium, *Micrococus luteus* (MIC 200 μg/ ml), but has no inhibitory effect on other bacteria tested (e.g., MIC> 400 μg/ml against *E. coli* K12, *Streptomyces albus* G, *Staphylococcus aureus*). In addition, with a radial diffusion assay, Klich found that Iturin A had marked antifungal activity against phytopathogens and human pathogens such as *A. flavus* (Klich *et al*. 1991); most fungi were inhibited at the concentration 7.7 μg/ml with no change in the zone of inhibition for several weeks.

Although this group of peptides is effective against dermatomycoses in humans and animals, they induce unacceptable levels of red blood cell lysis (Latoud *et al*. 1986).

Antifungal peptides with wide spectrum of antimicrobial activity

Most of the wide-spectrum AMP is enabled of lysing the membranes of the pathogens. Despite this non-specific mechanism, many of these peptides do not lyse mammalian membranes at concentrations of peptides that can inhibit the pathogen. Below are cited some examples of linear and cyclic antifungal peptides from different species (Matejuk *et al*. 2010).

Linear peptides

Small linear primarily α-helical peptides are the most common and well-studied group of antimicrobial peptides and include families such as cecropins (Steiner *et al*. 1981), magainins (Zasloff *et al*. 1987), and dermaseptins (Mor *et al*. 1991). Because α-helical amphipathic peptides differ in amino acid composition as well as in length and positive charge, their antimicrobial activity is likely determined by their global structural components rather than by the specific amino acid sequence (Dathe *et al*. 1999). The final common pathway for these α-helical amphipathic peptides is primarily disruption of the cell membrane; consequently, these

peptides have widespread activity against bacteria, fungi, and membrane-enveloped viruses. Nevertheless, accumulating data suggest that at least some of these peptides also have unique intracellular targets (Morton *et al*. 2007 and Marchand *et al*. 2006). In addition, many of them are able to lyse cancer cells at concentrations up to 10-fold lower than those required to lyse normal human cells (Cruciani *et al*. 1991).

Cecropins and cecropin-like peptides: These peptides have a broad spectrum of antimicrobial activity (bacteria and fungi) and have primarily been isolated from the hemolymph of silkworm moths (Merrifield *et al.* 1994 and Hultmark *et al.* 1993).These peptides range from 29 to 42 amino acids in length and form α-helices in hydrophobic environments such as the plasma membrane. Cecropin A at its microbicidal dose does not affect mammalian cells and numerous studies have shown that this peptide can be administered safely to animals (Moore *et al*. 1994 and Reed *et al*. 1997). At concentrations between 25 and 100 μg/ml cecropin A effectively killed *Aspergillus* spp., and at concentrations of 12.5 μg/ml, the peptide effectively killed *Fusarium moniliforme* and *F. oxysporum* (De-Lucca *et al*. 1998a and De-Lucca *et al*.1998b).

Magainins: A family of cationic amphipathic peptides that range between 21 and 26 amino acids in length and are rich in glycine and serine residues were isolated from the skin of *Xenopus laevis* (African frog) (Zasloff *et al*. 1987; Giovannini *et al*. 1987 and Terry *et al*. 1988). The magainin family of peptides includes magainin I, II, 2, PGLa peptides, xenopsin and the caerulein precursor fragment (Bevins *et al*. 1990; Sures *et al*. 1984 and Gibson *et al*. 1986). Some of these family members including magainin 2 and PGLa may form heterodimers, which increases their ability to permeabilize the membranes of pathogens (Hara *et al*. 2001). In addition to cidal activity of magainins against Gram-negative and Gram-positive bacteria and protozoa, magainins have antifungal activity against *Candida* spp., *C. neoformans, and S. cerevisiae.* Magainin 2 is particularly active against *C. neoformans* (MIC, 6.25 μg/ml) with greater activity than three other cationic peptides (Giacometti *et al*. 1999). Although Magainin 2 has moderate activity toward *C. albicans* (MIC > 80 μg/ml), it potently inhibits *C. glabrata* (MIC, 25.0 μg/ml), *C. tropicalis* (MIC, 12.5 μg/ ml), and *C. krusei* (MIC, 12.5–25.0 μg/ml) (Zasloff *et al*. 1987 and Giacometti *et al*. 1999).

Bombinin-H and bombinin-like peptides: Isolated from skin of *Bombina genu* (Rozek *et al*. 1988 and Simmaco *et al*. 1991) are glycine-rich, weakly cationic peptides with their C-terminal amidated amino acid (Simmaco *et al*. 2009). In addition to its inhibiting bacteria, bombinin-like peptides (BLP-1, 3) were active against fungi, especially *C. albicans* (MIC, BLP-1, 3–0.4 μM). Importantly, these peptides have little hemolytic activity (<10%, 15 μM). Bombinin-H peptides have varied antimicrobial and hemolytic activity (Mangoni *et al.* 2008 and Simmaco *et al*. 2009). Bombinins H2 and H4, which damage cell membranes of microbes, were found to be active against *C. albicans* (MIC, H2, 3.1μM; H4, 1.6 μM) , *C. guillermondii* (MIC, H2, 1.3 μM; H4, 0.7 μM), and *C. tropicalis* (MIC, H2, 1.1μM; H4, 0.6 μM) (Mangoni *et al*. 2000 and Simmaco *et al*. 2003). While bombinin-H2 induces 11% hemolysis, H4 induces 28 % hemolysis at 15 μM. Other bombinin H peptides, H6 and H7, with greater hydrophobicity, have lower antimicrobial activity and induce greater hemolysis than do H2 and H4 (Mangoni *et al.* 2008 and Simmaco *et al*. 2009).

Dermaseptins (S and B): These were identified in the skin of tree frogs of the genus *Phyllomedusa* (Mor *et al*. 1991). By interfering with lipid layers, which led to osmotic imbalance, dermaseptins lysed a wide spectrum of microorganisms. In addition to their antibacterial, antiviral, and anti-protozoa activity, they are cidal to pathogenic fungi (Lorin *et al*. 2005 and Hernandez *et al*. 1992) including yeasts and some filamentous fungi (*A. fumigatus*) (Mor *et al*. 1994). For example, a synthetic dermaseptin s1 analog, a 16- mer peptide, shows marked activity against *C. albicans* (MIC, 5.8 μM), and notably this analog had little hemolytic activity (Savoia *et al*. 2008).

Indolicidin: These are tryptophan-rich antimicrobial peptides that also belong to the cathelicidin family (Lawyer *et al*. 1996 and Selsted *et al*. 1992). Indolicidin and tritrpticin are expressed in neutrophils (Falla *et al*. 1996) of cow and pigs, respectively. Unlike the α-helical peptides previously discussed, these tryptophan-rich peptides have an extended wedge shape conformation in hydrophobic environments such as the plasma membranes (Lawyer *et al*. 1996; Selsted *et al*. 1992 and Yang *et al*. 2002). Indolicidin is biologically active against *S. aureus* and *E. coli* (Selsted *et al*. 1992), indolicidin has potent antifungal activity against *C. neoformans*

(MIC, 2–4 μg/ml) and good to moderate activity against *Candida* spp. (MIC,8–32 μg/ ml) with the exception of *C. guillermondii* (MIC>32 μg/ ml) (Benincasa *et al*. 2006).

Tritrpticin: A 13-amino acid peptide (VRRFPWWWPFLRR) containing 3 tryptophans (23%), 4 arginines (30%), and 2 prolines (15%). Although no *in vivo* studies have been performed to test the efficacy of tritrpticin, the peptide demonstrated weak activity toward *C. albicans* (MIC, 1000 μg/ ml) and *A. fumigatus* (250 μg/ ml) (Lawyer *et al.* 1996).

Kaxins: These are synthetic cationic antimicrobial peptides that have a non-amphipathic hydrophobic core segment (Stark *et al*. 2002 and Burrows *et al*. 2006). By inserting lysines at the N-terminal end and creating D-enatiomers peptides, Burrows and colleagues developed kaxins with potent candidacidal activity with little lysis. One kaxin, dF21-10K (kkkkkkkkkkaafaawaafaa-NH2), showed MIC between 16 and 64 μg/ ml against all fluconazole-sensitive and resistant *Candida* spp. and strains (*C. albicans, C. dubliniensis, C. glabrata, C. guillermondii, C. krusei, C. lusitaniae, C. parapsilosis* and *C. tropicalis).* Notably, dF21-10K showed marked activity with complete killing against biofilms formed by *C. albicans* or *C. tropicalis.* Although elimination of biofilms by antifungal molecules usually requires concentrations 30–2000 times more than their MIC (100), dF21-10K eradicated biofilms at only 10 times their MIC (Burrows *et al.* 2006).

Cyclic peptides

The vast majorities of broad spectrum cyclic antimicrobial peptides contain between 1 and 4 disulfide bonds and adopt β-sheet enriched structure including β -hairpin, β -sheet, or a-helix/beta-sheet mixed structures (Matejuk *et al*. 2010). Most of these AMP contain open-ended cyclic structures formed by internal disulfide bonds but θ-defensins, in addition to internal bonds, form closed-ended cyclic structures. With peptides such as defensins that contain multiple disulfide bonds, formation of the correct bond remains a challenge for developing peptide technologies (Matejuk *et al*. 2010).

Thanatin: Thanatin is an inducible and nonhemolytic 21-amino acid peptide isolated from the insect, *Podisus maculiventris.* Compared to other arthropod AMPs, thanatin has broad-spectrum antimicrobial activity. Thanatins are fungicidal against several phytomycotic diseases (e.g., MIC, *N. crassa, Botrytis cinerea, Nectria haematococca, Trichoderma viride, Alternaria brassicol* and *Fusarium culmorum* < 5 μM), and two pathogenic fungi in humans (MIC, *A. fumigatus*, 10–20 μM; *T. mentagrophytes*, 20–40 μM) (Fehlbaum *et al*. 1996). These peptides have no activity against yeasts such as *S. cerevisiae* or *C. albicans*. Interestingly, close homologs of thanatin isolated from skin secretions of frogs do have activity against *S. cerevisiae* and *C. albicans* (MIC, Brevinin-1E, 4.7 μM) (Simmaco *et al*. 1994).

Protegrins: These are approximately 2 kDa cysteine-rich β-sheet peptides found in porcine neutrophils. Similar to LL-37 and indolicidin, protegrins belong to the cathelicidin family of peptides, but unlike these two linear peptides, protegrins are cyclic antimicrobial peptides. Protegrins contain 16–18 amino acids and have 2 disulfide bridges which are essential for their antimicrobial activities, especially at physiological salt concentrations (Daly *et al*. 1999 and Shafer *et al*. 1998). Protegrins display broad-spectrum activity against bacteria, fungi, protozoa, and viruses (Tang *et al*. 1999, Shafer *et al*. 1998, Yasin *et al*. 1996a and Yasin *et al*. 1996b) and their primary mechanism of microbial action is due to lysis of the microbial membrane (Tam *et al*. 2000). Cho and colleagues examined the anticandidal activity of protegrins 1–5 (Cho *et al*. 1998), and found that the protegrins 1–3 and 5 had greater anticandida activity (MIC range, 2.50–2.85 μM) compared to protegrin 4 (MIC, 4.78 μM).

Mammalian defensins: A family of cationic peptides containing six highly conserved cysteine residues with three disulfide bridges (Ganz *et al*. 1994 and Owen *et al*. 2004) that are divided into three subfamilies: α-, β- defensins are found in many mammalian species and θ-defensins in *Rhesus macaques*. The α- and β-defensins differ in amino acid sequence and in the location of disulfide bonds. These mammalian defensins usually have antimycotic properties against *C. albicans* (Lehrer *et al*.1988 and Scott *et al*. 2000).

Syringomycins and related peptides: A group of cyclic lipopeptides, called lipodepsinonapeptides, produced by *P. syringae*. This group of peptides induces ion channels that affect membrane function, including membrane potential, protein phosphorylation, and H+-ATPase activity (Feigin *et al*. 1996 and Zhang *et al*. 1986). The family members (syringomycin E, syringotoxin B, and syringostatin A) were effective *in vitro* against a number of isolates of *Candida* spp. *(*MIC, 2.5–25 μg/ ml) and *C. neoformans* (MIC, 0.8–10 μg/ ml). Generally cyclic peptides were more effective against yeasts than against filamentous fungi. However, syringotoxin B was the least effective antifungal cyclic peptide. In a subsequent study, syringomycin E was found also to be effective in treating vaginal candidiasis in mice (Sorensen *et al*. 1996).

Antimicrobial peptides those are proteolytic fragments of proteins

The majority of peptides derived from proteins such as lactoferrin and pepsinogen A have broad-spectrum antimicrobial activity. For example, hydrolysates of lactoferrin have marked activity against Gram-negative and Gram-positive bacteria (Tomita *et al*. 1991).

Lactoferrin: An iron-binding antimicrobial glycoprotein (78 kDa) that is present in neutrophil granules, in breast milk, and on many mucosal surfaces (Nibbering *et al*. 2001 and Lupetti *et al*. 2000). There are two mechanism by which lactoferrin is thought to inhibit fungal and bacterial growth, by depriving iron which is essential to growth of fungi and generation of antifungal peptides from proteolytic enzymatic digestion of lactoferrin. Iron-binding properties of lactoferrin were reported to be responsible for the inhibition of *C. albicans* (Kirkpatrick *et al*. 1971).

CHAPTER 3

MATERIALS & METHODS

Chapter 3 *MATERIALS & METHODS*

3.1. Isolation and Characterization of Selected Antimicrobial Peptide (AMP) Producing Strains.

3.1.1. Sampling Sites

The extremophilic isolates from different areas of Arctic and Antarctic regions were collected from National Center for Arctic and Ocean Research (NCAOR) Sada Headland, Goa. Sediment cores from different sites at Lake Priyadarshini, Schirmacher Oasis (70°45 47.3 S', 11°44 22.8E'), East Antarctica were sampled by helicopter method (Singh *et al*. 2004), using a Hydro-Bios gravity core sampler. Cores collected from a depth of 6.5 m were found to be 80 cm in length. After dredging, the cores were cut into 2-cm slices starting from the top, labelled and stored in sterile polythene bags. Soil, faeces and feather samples were collected from penguin rookeries of Larsemann Hills, East Antarctica (69°21.68'S, 76º07.76'E; 69°21.68'S, 76º07.70'E and 69º22.433'S, 76°08.940'E). In Arctic, Ny Alesund, water samples were collected from a glacier mouth (78°53.703'N, 12°02.475'E), glacier stream (78°53.75'N, 12°02.502'E), sea convergence (78°56.106'N, 11°51.854'E) and permafrost soil samples (78°55.165'N, 11°52.660'E). The collected water and soil samples were stored at 4°C until processed (Shekh *et al*. 2011).

3.1.2. Isolation and Growth Characteristics

A total of 100 µl of each sample was spread on 1/10 Zobell marine agar (ZMA) and 1/10 malt, glucose, yeast extract, peptone (MGYP) agar plates (Table 2). In a separate experiment, samples were filtered using 0.4µm Millipore membranes. The filter membranes were placed upon 1/10 ZMA, 1/10 nutrient agar media and incubated at 4 and 15°C for 1–2 weeks. Appropriately diluted penguin rookery samples were also plated on two types of media mentioned earlier in

order to recover most of the representative bacteria at two different temperatures (5º and 15°C) suitable for psychrophilic and psychrotolerant species.

After the appropriate incubation period, all plates were screened for colonies. Isolated colonies were counted, picked and transferred to MGYP broth with 1.9% NaCl and further incubated at 5 and 15°C until sufficient growth was observed.

In order to screen faeces and feather samples from the Penguin rookery and Arctic water, modified Trypticase broth (mTSB, E–Merck, Table 3) was used as a medium for the production of antimycotic/antimicrobial substances. As many as 240 isolates were screened from the soil and fecal samples of penguin rookery and Arctic glacier melt water and sea–convergence of Arctic region at Ny Alesund, Norway. All samples were spread on MGYP agar, Himedia, India, Table 2, with 1.9% NaCl (Gilbert *et al*. 2004), after appropriate dilutions and subsequently individual colony grown on the MGYP agar were transferred into the modified TS broth. All the incubations were carried out at 15ºC for 48 hours.

Materials

Table 2. Composition of MGYP broth for 1 liter medium

Malt extract	3.0 gm
Glucose	10.0 gm
Yeast extract	3.0 gm
Peptone	5.0 gm
pH	6.4±0.4
Agar bacteriological	1.8-2.0 %

Table 3. Composition of mTS broth for 1 liter medium

Casein enzymatic hydrolyzate	17.0 gm
Papaic digest of soyabean meal	3.0 gm
NaCl	5.0 gm
Dipotassium phosphate	2.3 gm
Dextrose	2.5 gm
Yeast extract	2.5 gm
pH	7.4 ± 0.2

3.1.3. Test for Antimicrobial Activity

The wild-type isolates were screened by examining the production of antimicrobial/antimycotic substances against various multidrug-resistant yeast strains (namely, *C. albicans* NCIM 3471, NCIM 3557, MTCC 3958, MTCC183, MTCC 227, MTCC 7315 NCIM 3129 (*C. krusei*), SC 5314 brought from Jawaharlal Lal Nehru University (JNU), New Delhi, wild type *C. albicans* (WI) and diabetic isolate (DI) from Goa. Initially, the distinct, isolated colonies from all MGYP or nutrient agar plates were randomly picked, transferred onto modified trypticase soy broth (mTSB) and incubated for 48h at 15°C. Cell-free supernatants (CFS) were prepared by centrifuging at 10,000 rpm for 30 min at 4°C. CFS was stored at – 20°C until further use.

8-mm wells were made using a sterile well borer on solidified MGYP agar (pH 6.4 ± 0.4) plates. A total of 100 µl of CFS was added to each well, and the plates were incubated at 37°C for 12 h for diffusion. An aliquot of 100 µl of appropriately diluted (1:1 dilution), freshly grown indicator organism (*C. albicans*) was spread uniformly with a sterile, bent glass rod. Plates were incubated at 27°C for 2–3 days and then were inspected for clear zones of inhibition around the wells, and the zone diameter was measured (Shekh *et al*. 2009). For the bacterial strains used as test organisms, instead of MGYP, the medium brain heart infusion (BHI) was used.

3.1.4. Morphological and Biochemical Characterization

3.1.4.1. Morphological characteristics

Motility, acid production, gas production and Gram staining was performed for the selected antimicrobial substance producing isolates named (AGM108–5, AGM111, APR 210, APR 211).

3.1.4.2. Sugar fermentation test

The sugar fermentation profile of the most promising isolate (APR210), was checked by using BK009 HiCarbohydrate kit Himedia, with glucose, lactose, salicin, mannitol, xylose, maltose, fructose, dextrose, galactose, rafinose, trihalose, malibiose, sucrose, arabinose, mannose, inulin, sodium gluconate, glycerol, dulcitol, inocitol, sorbitol, adonitol, arabitol, erythritol, α-methyl d-glucoside, rhamnose, cellobiose, melezitol, α-methyl d-mannoside, xylitol, ONPG, esculin hydrolysis, d-arabinose, citrate utilization, malonate utilization and sorbose.

3.1.4.3. Antibiotic sensitivity test

The antibiotic sensitivity test of the selected isolates were performed by using ampicillin, ampicillin/ cloxacin, tetracyclin, chlortetracycline, chloramphenicol, streptomycin, rifampicin, vancomycin, novobiocin, kanamycin and neomycin discs (Himedia, India).

3.1.4.4. Salt tolerance, different incubation temperature, pH, catalase and oxidase tests

Growth of selected isolates were checked in presence of different concentration of salts (0–9%), at different incubation temperatures (0, 5, 15, 18 and 22ºC), different pH values (7.0 –9.0), in de Man, Rogosa Sharpe (MRS) broth, pH 6.6±0.2. Catalase and oxidase tests were also performed for the selected isolates.

3.1.4.5. Hemolytic activity assay on the blood agar plates

The most promising antimicrobial substance producing isolate (APR 210) was checked for hemolytic activity assay on the sheep blood agar plates having 5% defibrinated sheep blood. *Streptococcus pyogenes* MTCC 442 was used as control for β-hemolysis.

3.1.5. Growth of *E. faecalis* APR 210 Strain in Potassium Tellurite

E. faecalis was grown in mTS broth overnight at 14°C in BOD incubator. More than 24 hours of the culture was used for inoculation in freshly made mTS broth having 0.04% potassium tellurite. An overnight grown (OD 0.6) culture of *Escherichia coli* and *Staphylococcus aureus* were used as negative and positive controls respectively.

Procedure

1. Inoculated the mTS broth having 0.04% potassium tellurite with freshly grown *E. faecalis* at the rate of 1.0 % as well as *E. coli* and *S. aureus.*
2. Incubated at 14°C for 2–3 days.

3.1.6. Fatty Acid Methyl Ester (FAME) Analysis

The selected isolates were subjected to the Fatty Acid Methyl Ester (FAME) analysis to find the composition of the cell walls. For extraction of fatty acid methyl esters, the fatty acids were prepared from 40 mg wet cell material harvested from a culture on TS agar (30 g TS, 15 g agar; Hi-Media, India) incubated for 5 days at 15 °C. Whole-cell fatty acids were determined as described by (Bozal *et al.* 2002).The extraction procedure and instruments were described previously (Pikuta *et al*. 2003).

3.1.7. DNA Sequencing and Identification of Selected Bacterial Strains

16S rDNA gene sequence typing was performed to identify the selected polar isolates to the species level. Genomic DNA was extracted from bacterial isolates using a KT 83B kit (cat no. 105604, GeNeI, Bangalore, India). The 1.5-kb 16S rDNA gene was amplified by PCR using universal primers and high-fidelity PCR polymerase. The length of primers was 20 nts (forward primer 5'-AGAGTRTGATCMTYGCTWAC-3', reverse primer 5'-CGYTAMCTTWTTACGRCT-3'), and the amplicon generated was 1,500 bp long. The PCR conditions were 94°C for 5 min, 94°C for 30s, 55°C for 30s, 72°C for 2 min and finally 72°C for 5 min. The number of cycles was 35. The PCR product was checked on a 1% agarose gel and purified using a Qiagen purification kit (Hilden, Germany). The purified product was cloned into a TA vector (KT 63A, cat no. 107416, GeNeI, Bangalore, India) and bidirectionally sequenced with vector-specific forward, reverse and internal primers.

The sequence data obtained for 16S rDNA genes were entered into the GenBank database and assigned accession number (HM481246). The nearest homologues for each isolate were determined by comparison with the database using the BLAST search tool (Altschul *et al*. 1990 and Shekh *et al*. 2011).

3.1.8. Phylogenetic Tree Construction of Selected Strain

The sequences were obtained with the Applied Biosystems sequencer model ABI 3100. Sequence data was aligned and analyzed for finding the closest homologs and percent similarity for the microbes with the help of website *www. EzTaxon.org* as well as NCBI BLAST.

The sequence data for the 16S rDNA genes was entered into the Genbank database and each sequence assigned an accession number. The nearest published relative for each isolate was determined by comparison with the database using the BLAST search tool. Similarity searches of the sequences obtained were performed using BLAST (Altschul *et al*. 1990). Sequences were then aligned to their closest related sequences determined from the BLAST searches using the ClustalW program (Thompson *et al*. 1994). The aligned sequences were then checked manually

for gaps. The DNADIST program was used to compute pair wise evolutionary distances for the aligned sequences by applying the Kimura two-parameter model (Kimura *et al*.1980). The phylogenetic distance tree was constructed in NJplot software using the neighbor joining method (Saitou *et al*.1987).

3.2. Characteristics of Indicator Organisms

3.2.1. Media and Growth Parameters

Most of the indicator organisms were procured from Microbial Type Culture Collection (MTCC) Chandigarh and National Collection of Industrial Microorganisms (NCIM) Pune. Two wild-type *Candida albicans* designated as DI and WI were isolated from subjects of Goa and Dehradun. The indicator organisms used in cut-well agar assay was propagated in MGYP agar and broth (pH 6.6 $\pm$ 0.2). The composition of MGYP is given in Table 2. The indicator organisms were grown at the temperature 25 and 35°C based on MTCC and NCIM guidelines and the wild type *Candida* were grown at 37°C. All microbiological media components were purchased from Hi-Media, Mumbai, India. All strains were stored in appropriate media with 20 % glycerol at –80 °C.

3.2.2. Identification of Wild type *C.albicans* (DI and WI)

Genomic DNA of wild type indicator strains was isolated from the pure culture. The ~ 1.5 kb rDNA fragments (small subunit ribosomal RNA, 18S) were amplified using high–fidelity PCR polymerase. The length of primers was 20 nts (forward primer 5'-AGAGTRTGATCMTYGCTWAC-3', reverse primer 5'-CGYTAMCTTWTTACGRCT-3'), and the amplicon generated was 1,500 bp long. The PCR conditions were 94°C for 5 min, 94°C for 30 s, 55°C for 30 s, 72°C for 2 min and finally 72°C for 5 min. The number of cycles was 35. The PCR product was checked on a 1% agarose gel and purified using a Qiagen purification kit. The PCR product was sequenced by primer walking. The sequence data was aligned and analyzed to identify the isolate and its closest neighbors.

3.2.3. Antifungal and Antibacterial Susceptibility Testing of Indicator Organisms

The indicator organisms *C. albicans* (MTCC 183, MTCC 3958, MTCC 7315, MTCC 227, MTCC 7315), *C. krusei* (MTCC 3129), wild type Candida (WI), Candida diabetic isolate (DI) and *C.albicans* (SC 5314) were checked for sensitivity and resistance against several antifungals Amphotericin B (20 mcg/disc), Itraconazole (10 mcg/disc), Nystatin (100u/disc), Clotrimazole (10mcg/disc), Fluconazole (25 mcg/disc), Miconazole (50mcg/disc),Voriconazole (1 mcg/disc) and some antibacterial antibiotics. The sensitivity and resistance was determined based on zone of clearance according to the zone size interpretive chart provided by the Himedia, India.

3.3. Antimicrobial Protein/Peptide Production by *E. faecalis* APR 210

3.3.1. Screening of APR 210 for Antimicrobial Potential

The antimicrobial substance production was determined by inoculating 1% (10^9 CFU/ml) of an overnight culture of *E. faecalis* APR 210 in mTS enriched broth and incubated at 14°C under uncontrolled pH conditions without agitation. After 48–50 hours of incubation the cell free supernatant (CFS) was prepared by centrifuging at 12,000 rpm for 30 min at 4°C. CFS was stored at –20°C until further use.

In order to test the antimicrobial activity by cut-well agar assay, 8-mm wells were made using a sterile well borer on solidified MGYP agar (pH 6.6 ± 0.2) plates. A total of 100 µl of CFS was added to each well, and the plates were incubated at 37°C for 12 h for diffusion. An aliquot of 100 µl of appropriately diluted (1:1 dilution) of freshly grown indicator organism (*C. albicans*) was spread uniformly with a sterile, bent glass rod. Plates were incubated at 27°C for 2–3 days and then were inspected for clear zones of inhibition around the wells, and the zone diameter was measured. For the bacterial strains used as test organisms, instead of MGYP, brain heart infusion (BHI) agar and broth (pH 7.4 ± 0.2) were used (Shekh *et al*. 2011).

3.3.2. Optimization of Physical and Chemical Parameters for Anti-*Candida* Protein (ACP) production by *E. faecalis*

Parameters such as optimum incubation time, different media, selected media pH, incubation temperature, inoculum size, salt concentration and media composition were optimized. Further the sets of experiments were performed at different temperatures 5, 10, 15, 20, 25, 30, 35, 40 and 45°C. The flasks were incubated for 120 hrs and samples were collected at 8, 12, 16, 24, 36, 48, 72, 96 and 120 hrs and analyzed.

The growth as well as biological activity of antimycotic or anti-*Candida* protein (ACP) producing culture was determined in different media having nutrient broth NB, BHI, methyl red-Vogues-Proskauer (MR-VP), MGYP, MTSB and TSB. Optimization of media pH was done with mTS broth adjusted to pH 2.0, 4.0, 6.0, 7.0, 8.0, and 10.0, inoculated with 1.0 % of ACP producing culture grown overnight at 14°C. Inoculum size was optimized for 0.5, 1.0, 2.0 and 5.0% of inoculum at 14°C and constant pH 7.2 ± 0.2.

Sodium chloride at varying concentrations (0, 1, 2, 3, 4, 5, 6 and 7% w/v) were added in mTS broth medium and incubated at 14º and 37°C for their effect on ACP production. The composition of mTS broth is shown in Table 3.

3.3.3. Arbitrary unit (AU/ml) determination of ACP

ACP activity in the cell free supernatant(CFS), dialyzed concentrate (DC), biologically active fractions collected after ion exchange and gel filtration chromatography was determined by the agar well diffusion assay and critical dilution method, currently used for the assay of ACP (De-Vuyst *et al.*1996). Serial twofold dilutions of cell-free culture supernatant containing ACP were spotted (10 µl) on agar plates containing overlay of fresh cultures of a sensitive strain or the cell free culture supernatant (50 µl) was loaded into the well. These overlay cultures were prepared by propagating fresh cultures to an optical density at 600 nm of 0.45, and adding 100 µl of the cell suspension to 3.5 ml of overlay agar. Overlaid agar plates were incubated for 24–48 h at the appropriate temperature. The ACP activity was defined as the reciprocal of the highest dilution

that demonstrated complete inhibition of the indicator lawn, and was expressed in activity units (AU) per milliliter of culture medium.

$$\text{Antimicrobial Activity (AU/ ml)} = \frac{\text{The highest dilution provided inhibition zone} \times 1{,}000}{\text{Volume (µl)}}$$

3.3.4. Study of Growth Pattern of ACP Producing *E. faecalis*

The kinetics of ACP production was determined by inoculating with 1.0% (10^9 CFU/ml) of an overnight culture of *E. faecalis* in mTS enriched broth and incubating at 14°C under uncontrolled pH conditions without agitation. At 4 hour intervals, samples were collected to determine the optical density at 600 nm as well as pH. The antimicrobial activity was determined assaying serial two fold dilutions of cell free culture supernatants against *C. albicans* MTCC 183 (10^8 CFU/ml). The antimicrobial titer was defined in arbitrary units (AU/ml) as the reciprocal of the highest dilution showing inhibition around the well (5.0 mm) (Shekh *et al.* 2012).

3.3.5. Protein Quantification

Protein concentrations were determined by Bradford dye binding method (Bradford, 1976) using a protein assay kit from Biorad Reagent Bio 46 (Chromous Biotech, India). Assay was performed in glass tubes.

Materials

Bovine serum albumin (BSA), sterile double distilled water, Bradford reagent, dialysed concentrate and CFS of *E. faecalis*

Bradford Reagent:

- 20 mg Coomassie Brilliant Blue G-250
- 10 ml 95% ethanol
- 20 ml 85% phosphoric acid
- Whatmann No.1 filter paper

Stock concentration of BSA: 1mg/ml

10ml of 1mg/ml BSA was prepared by adding 10 mg of BSA in 10 ml of sterile double distilled water.

Working concentration of BSA: the different concentrations of BSA like 25, 50, 75, 100, 125, 150 and 175µg/ml were used to prepare the standard curve.

Procedure

1. Pipetted 0, 2, 5, 10 and 20 µl of BSA (1mg/ml) into individual tubes.
2. Pipetted upto 20 µl of unknown samples into separate tubes.
3. Bradford reagent (200 µl) was mixed with 20µl of distilled water was used as blank.
4. Added 200 µl of Bradford reagents into all tubes containing standards or samples.
5. Added double distilled water to all tubes to bring the final volume to 1ml and mixed well.
6. The absorbance was determined at 595 nm within 15-16 min using UV-Visible spectrophotometer (Shimadzu), were plotted for quantification for total protein.

Table 4. Preparation of Protein Samples:

	Concentrate		Filtrate	
	C1	C2	F1	F2
Protein(µl)	3	5	100	150
D.D.water(µl)	997	995	900	850
Bradford Reagent(µl)	2000	2000	2000	2000

As given in the Table 4, BSA and other protein samples were prepared and O.D was measured.

3.4. Characterization of Anti-*Candida* Protein (ACP)

3.4.1. Antimicrobial Activity against different Indicator Organisms

The anti-*Candida* activity of the CFS, dialyzed concentrate of *E. faecalis* was assayed against the different yeast *C. albicans* MTCC 183, MTCC 3958, MTCC 7315, NCIM 3471, NCIM 3129 as well as wild type (WI), diabetic isolate (DI), and *C.albicans* (SC 5314) using the agar well diffusion assay method as described previously. After 24–48 h of incubation the zone of clearance was checked. To determine the titer of the antifungal activity, serial twofold dilutions of the extracts were performed. The anti-*Candida* activity was expressed as activity units AU/ml corresponding to the reciprocal of the highest dilution causing inhibition of the yeast growth (Shekh *et al.* 2012).

3.4.2. Biochemical Characterization of ACP

3.4.2.1. Effect of hydrolytic enzymes on the biological activity of ACP

Sensitivity of the (ACP) to proteolytic enzymes, trypsin, lipase, lysozyme, pepsin, α amylase, pronase E, and proteinase K, using 50 mM Tris pH 8.0, 20mM Sodium Phosphate pH 7.0, 50mM Sodium Phosphate pH 7.0, 20mM Tris HCl pH 2.0-4.0, 50mM Sodium Phosphate pH 7.0, 10mM Sodium Phosphate pH 7.0 and 50mM Tris HCl pH 7.5 respectively. Each enzyme was dissolved in appropriate buffer and added to the ACP solution for a final concentration of 1 mg/ml, following incubation at 37 °C for 2 h. Four wells were made in a petriplate having enzyme in appropriate buffer treated with the ACP in first well, appropriate buffer in second well, only ACP in third well and enzyme buffer in fourth well. After complete diffusion the indicator organisms *C. albicans* MTCC 7315 and MTCC 183 were spread uniformly over the plate and the zone of clearance was determined after 48 h of incubation (Shekh *et al*. 2012).

3.4.2.2. Effect of different pH on the activity of ACP

To evaluate the effect of pH on ACP activity, the supernatant pH levels were adjusted between 2.0 and 10.0 using 1 N HCl and 1 N NaOH. The pH stability of dialyzed concentrate was assayed at pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and at room temperature (25°C) after 2 h of incubation. The remaining activity (AU/ ml) was measured by bioassay. Untreated samples were used as the controls (Shekh *et al*. 2012).

3.4.2.3. Thermal stability of ACP

Temperature stability was evaluated by incubating the CFS at various temperatures having 60°C for 90 min, 90°C for 20 min, 100°C for 20 and 30 min and autoclaved. Residual anti- *Candida* activity was determined by well diffusion assay against *C. albicans* (Shekh *et al*. 2012).

3.4.2.4. Effect of different organic solvents on the activity of ACP

The sensitivity of dialyzed concentrate of ACP was tested in the presence of several organic solvents namely (methanol, ethanol, isopropanol, hexane, formaldehyde, chloroform, acetone and acetonitrile) at a final concentration of 25% (v/v). After 2 h of incubation at 37°C, the organic solvents were evaporated using speed vac system (Martin Christ), and the residual antimicrobial activity was determined. An untreated dialysed concentrate sample was taken as control (Shekh *et al*. 2012).

3.4.2.5. Effect of surfactants

Effect of various surfactants including Triton X-100, Tween-20, SDS, urea, EDTA, PMSF, DTT (1.0 % each) on the dialyzed concentrate was also tested, and to ensure whether the antifungal activity is due to the oxidation state of cysteine residue, β-mercaptoethanol (1.0 and 2.0 mM) was used. The heat-treatment at 80°C was given for 10 mins (Hastings *et al*. 1991).

3.4.2.6. Effect of different storage temperature

In order to determine the stability, the CFS, dialyzed concentrate and purified ACP sample was stored for 1 year at low temperatures (4, −20 and −80 °C) and antimicrobial activity was compared with the fresh purified preparation.

3.5. Partial Purification of ACP

3.5.1. Preparation of Cell Free Supernatant (CFS)

The producer strain at the rate of 1.0 % (10^8 CFU/ ml) was inoculated into mTS broth and incubated at 14°C in BOD incubator (Remi India) without agitation. After 48hrs of incubation the CFS was made by centrifugation (Eppendorf Centrifuge 5810R) at 12000 rpm, 4°C for 30 minutes and was carefully transferred in another sterile tubes/conical flasks without disturbing the cell pellet. The CFS was filtered through 0.45 µm membranes and stored at −80°C for further application.

3.5.2. Precipitation of ACP by Ammonium Sulfate Fractionation and Dialysis

The ACP having CFS was subjected to sequential ammonium sulphate precipitation to achieve 30, 50 and 85% saturation at 4°C, by slow addition of the salt, with constant and gentle stirring, after addition of all the salts the protein solution was stirred 4h at 4°C. The precipitated proteins were pelleted out by centrifugation at 12000 rpm, 4°C for 30 mins. The protein pellet was dissolved in sterile 20 mM sodium phosphate buffer pH 8.0, and dialysed using a 10 kDa MWCO membrane (Slide-A-Lyzer Dialysis Cassette, Thermo Scientific), overnight, at 4°C, against the same buffer. The crude preparation was then stored at -80°C for further analysis (Shekh *et al*. 2011).

3.5.3. Ion Exchange Chromatography

A column was prepared manually (12 cm length and 1.5 cm diameter) and packed with 10 ml DEAE Sepharose (weak anion exchanger, GE Healthcare) matrix. The packed column was equilibrated with 20 mM sodium phosphate buffer pH 8.0, (buffer A) and 6 ml of dialysed concentrated biologically active crude protein was loaded on top of the column. Linear gradient of 0 to 0.25 M NaCl, including 20 mM sodium phosphate buffer, pH 8.0 (buffer B) was applied. As many as sixty fractions of 3 ml were collected, and all the fractions were tested for anti-*Candida* activity. The absorbances of all fractions were recorded at 280 nm. All the fractions

with antifungal activity were pooled and subjected to ultra filtration (Pall Science) for concentration and removal of salts (Shekh *et al*. 2012).The pooled concentrated (10 kDa ultra filtration membrane Pall Science) 5X fractions was tested against *C. albicans* SC 5314 strain.

3.5.4. Gel Filtration using Sephadex G-75

Anti-*Candida* protein of *E. faecalis* was purified further by gel filtration using Sephadex G-75 column, passing continuously 20 mmol sodium phosphate buffer (pH 8.0). Three grams of Sephadex G-75 were soaked in 200 ml of sterile distilled water and washed three times for removing fine particles, and then dissolved in 20 ml of sterile distilled water and poured in a 1.0 x 50 cm column. Void volume was determined by passing blue dextran (2000 kDa) through the column. The ion-exchange fractions showing biological activity were pooled (2.0 ml) and loaded onto the gel filtration column. The above mentioned buffer was used to elute the sample fractions each of 1.5 ml those were collected at a flow rate of 65ml/hr and recorded at 280 nm using UV-Visible spectrophotometer (Shimadzu). Antimycotic assay for all fractions were performed against the indicator strains *C. albicans* MTCC 3958 and MTCC 183. The active fractions were pooled and subjected to ultrafiltration (desalting) using Pall Science membrane and freeze drying (lyophilization) for further analysis.

3.5.5. Molecular Weight Determination

To estimate the molecular mass of the bioactive peptides, Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) as well as Tricine native PAGE were carried out as described by Laemmli *et al*.1970 and Schagger *et al*. 1987.10·0%, SDS-PAGE was run using Technosource Microkin electrophoretic unit. Electrophoresis was conducted at a constant voltage of 100 V/ 20mA for 2.5 h. Standard weight (14.3 - 97.4 kDa) protein marker (Genei, Banglore, India) was run for size estimation. After electrophoresis, the gel was removed from plates and stained using Coomassie brilliant blue R-250 (Himedia, India) or silver staining.

Materials

Reagents for SDS – PAGE

1. 30% Acrylamide and N, N'-methylene bisacrylamide mix (w/v).
    a. Acrylamide 29.2 gm
    b. N,N'- methylene – bisacrylamide 0.8 gm
    c. Distilled H_2O 100 ml

    Made the volume upto 100 ml. Stored it in amber colored bottle at 4°C
2. 1.5 M Tris (pH 8.8)
3. 1.0 M Tris (pH 6.8)
4. SDS 10% in sterile distilled water (w/v).
5. Ammonium per sulfate 10% in sterile distilled water (w/v) was freshly prepared.
6. TEMED (N, N, N', N'– tetramethylene diamide) was added just before casting the gel.

10% SDS – PAGE Resolving gel	(20.0 ml)
1. H_2O	7.9 ml
2. 30% Acrylamide mix	6.7 ml
3. 1.5 M Tris (pH 8.8)	5.0 ml
4. 10% SDS	0.2 ml
5. 10% APS	0.2 ml
6. TEMED	0.006 ml

5% stacking gel	(5.0 ml)
1. ddH_2O	3.40 ml
2. 30% Acrylamide mix	0.83 ml
3. 1.0 M Tris (pH 6.8)	0.63 ml
4. 10% SDS	0.05 ml
5. 10% APS	0.05 ml
6. TEMED	0.005 ml

1X SDS – PAGE running buffer	(200 ml)
1. Tris	0.6 gm
2. Glycine	2.9 gm
3. SDS	0.2 gm

SDS – PAGE loading dye (3X) stock	(10.0 ml)
1. 1M Tris-Cl (pH- 6.8)	2.4 ml
2. 20% SDS	3.0 ml
3. Glycerol (100%)	3.0 ml
4. β – mercaptoethanol	1.6 ml
5. Bromophenol blue	0.006 gm

Running buffer (10X)	1000.0 ml
1. Tris base	30.3gm
2. Glycine	114.0gm
3. SDS	10.0gm

Protein sample 6X was mixed with 1X of loading dye, vortexed before boiling for 8 minutes and then again vortexed before loading in the well of acrylamide gel.

Coomassie brilliant blue staining solution	(250 ml)
1. Coomassie brilliant blue R -250	0.5 gm
2. Methanol	112.5 ml
3. Acetic acid	22.5 ml
4. H_2O	115.0 ml

Destaining solution	(1000 ml)
1. Methanol	150.0 ml
2. Acetic acid	100.0 ml
3. H_2O	750.0 ml

Resolving gel

1. Mixed the separating gel solution by adding 29.2% acrylamide 0.8% bis-acrylamide, 1X 1.5M Tris-CI, pH 8.8, and water to make 20 ml final volume for a Microkin electrophoretic unit in a flask. 200 µl of 10% ammonium per sulfate (APS, freshly prepared) was added and 8.0 µl TEMED just before polymerization.
2. Transferred the separating gel solution into the glass cassette using a pipette and was allowed to polymerize fully.

Stacking gel

1. 5% stacking gel solution was prepared by adding 0.83 ml of 29.2% acrylamide/0.8% bisacrylamide, 0.63 ml of 1.0M, 1X Tris-CI, pH 6.8, and 3.4 ml water. Add 50 µl of 10% APS and 5 µl TEMED to the solution and gently swirled to mix. Slowly pourded the stacking gel solution over the polymerized resolving gel.
2. Inserted a Teflon comb into the layer of stacking gel solution and was allowed to polymerize completly.

Loading the gel

1. Protein sample to be analyzed was diluted at least 3:1 (v/v) with 3X loading dye in a microcentrifuge tube and boil for 7 min at 100ºC. After boiling, cooled the sample to room temperature, and centrifuged for a few seconds to remove particulate matter.
2. Teflon comb was removed carefully without tearing the edges of the polyacrylamide wells.
3. 1X SDS/electrophoresis buffer was poured in electrophoretic apparatus.
4. Protein samples were loaded into the wells by carefully applying the sample as a thin layer at the bottom of the wells.

3.5.6. Different Staining Methods of Protein Bands

Coomassie Brilliant blue staining

1. The gel removed from the glass plates was washed 2 –3 times with milliQ water in a clean staining tray and filtered freshly prepared Coomassie brilliant blue stain (R–250) solution was used for staining.
2. After 6–8 hours of shaking the staining solution was decanted and the destaining solution was poured with continuous change and shaking.
3. When the protein bands were visible the change of destaining solution was discontinued.

Silver staining

1. Silver staining was done based on the protocol in Protein Analysis (Mortz *et al*. 2001).
2. Removed the gel from the plates and washed for 5 minutes in sterile milliQ water.
3. Incubated the gel in Fixer (40% ethanol, 10% acetic acid, 50% milliQ water) for 1 hour.
4. Washed the gel in sterile milliQ water for at least 30 minutes. Sensitized the gel in 0.02% sodium thiosulfate (0.04 g $Na_2S_2O_3$, 200 ml water) for only 1 min followed by washing the gel in milliQ water for 3×20 sec.
5. Incubated the gel for 20 min in 4°C cold 0.1% silver nitrate solution (0.2g $AgNO_3$, 200 ml milliQH_2O, 0.02% formaldehyde (added 40µl 35% formaldehyde just before use) followed by washing the gel in milliQ H_2O for 3×20 sec.
6. The gel was washed in milliQ water for 1 min keeping the gel in new staining tray.
7. Developed the gel in 3% sodium carbonate (7.5g Na_2CO_3 in 250 ml H_2O), 0.05% formaldehyde (added 125 µl 35% formaldehyde just before use).Changed the developer solution immediately when it turned yellow. Terminated the staining when the colour development was sufficient.
8. Washed the gel in milliQ for 20 seconds.
9. Terminated staining in 5% acetic acid for 5 min and stored the gel at 4°C in 1% acetic acid.

3.5.7. Direct detection of Biological Activity on Tricine PAGE

Tricine Native-PAGE (10 %) (Schagger *et al*. 1987), followed by a gel overlay was performed with active pooled fractions from gel filtration. After electrophoresis for 2 h at 20 mA, 2 duplicate gels were cut. One of the gels was silver stained. The other gel was fixed in 20% (v/v) isopropanol and 10 % (v/v) acetic acid for 30 min, rinsed with 500 ml of MilliQ water for 1 h, and placed aseptically on an MGYP plate. To identify the active peptide band, the Tricine gel containing pooled active fraction was overlaid by freshly grown *C. albicans* MTCC 3958. After the agar solidified, the plate was incubated at 37°C for 48–72 h until *C.albicans* grew uniformly over the plate or an inhibition zone was observed (Bhunia *et al*. 1987).

3.5.8. Mass Spectrometry and *de novo* Sequencing of Purified ACP

The purified antimicrobial peptide was analyzed by Matrix-Assisted Laser Desorption and Ionization–Time of Flight Mass Spectrometry (MALDI-TOF/MS) by using a 4000 Q TRAP Mass Spectrometer (Proteomics International, Nedlands Australia) equipped with an ion source with visualization optics and an N_2 laser (337 nm). Protein samples were Trypsin digested overnight and peptides extracted according to standard techniques (Bringans *et al*. 2008).All digestion reactions were done in 50 mM NH_4HCO_3 (pH 8.5) at room temperature and with an enzyme-to-peptide ratio of 1:40 (w/w). Peptides were analyzed by electrospray ionisation mass spectrometry using the Ultimate 3000 nano HPLC system [Dionex] coupled to a 4000 Q TRAP mass spectrometer (Applied Biosystems) with a capillary cap voltage of 1,750 V. Tryptic peptides were loaded onto a C18 PepMap100, 3 μm [LC Packings] and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v). MS/MS spectra were analyzed using PEAKS Studio Version 4.5 SP2 [Bioinformatics Solutions]. The mass data collected during LC/MS/MS analysis were processed, converted into mgf files, and compared against the Ludwig NR database by using a local MASCOT server.

The three most abundant peptides, preferably doubly charged ions, corresponding to each MS spectrum were selected for further isolation and fragmentation. The MS/MS scanning was performed in the ultrascan resolution mode at a rate of change in the m/z of 26.000/s (Shekh *et al.* 2012)

Sample preparation guideline

1. Used clean plates and containers for gel casting and staining.
2. While excising the bands, surface and new razor blades were washed with 70% ethanol or used a cleaned scalpel blade every time for cutting the gel.
3. Cut close to the stained portion of the gel to minimize the excess acrylamide.
4. Labeled clearly each tube and stored at 4°C for further use.

3.5.9. Transfer of Protein Bands to Polyvinylidene difluoride (PVDF) membrane (Immobilon-P 0.45 µm) and Staining

Materials

1X Transfer Buffer composition

1.	Glycine	28.8 gm
2.	Tris Base	6.04 gm
3.	Methanol	200 ml
4.	Milli Q water	1.6 l

Coomassie Brilliant Blue R 250 stain (100 ml)

1.	Coomassie brilliant blue R-250	0.10 gm
2.	Methanol	40 ml
3.	Acetic acid	1.0 ml
2.	Sterile MilliQ water	59 ml

Procedure

1. Ran 10% Tricine Native PAGE, after electrophoresis, separated the gel carefully and washed it in 1X Transfer Buffer for 30 min.
2. Soaked four to five sponges, two pieces of Whatman 3mm filter paper in Transfer Buffer. Soaked the PVDF membrane briefly in 100% (v/v) methanol followed by 1X Transfer Buffer. Assembled the "sandwich" on top of the open transfer cage in the following sequence: two sponges, one filter paper, the polyacrylamide gel, the membrane, one filter paper and two to three sponges.
3. Closed the transfer cage to complete the "sandwich" and immersed the assembly into the transfer chamber. Ensure that the cage was oriented such that the side of the gel contacting the membrane was facing the positive electrode.
4. Filled the chamber with Transfer Buffer and electrophorese the proteins from the gel to the membrane at 100mA for overnight.
5. After complete transfer, removed the PVDF membrane carefully, washed in sterile MilliQ water.
6. Stained with 0.1% coomassie brilliant blue R-250 for 30 seconds.
7. Destained with 40% methanol, 1.0% acetic acid until the bands visible and background clear.
8. Cut the concerned visible band with sterile blade, dried it keeping inside tissue papers and stored the PVDF membrane at 4°C.

3.5.10. N-Terminal Amino Acid Sequencing of Purified ACP

N-terminal amino acid sequencing was done in Iowa State University, Protein Facility, USA by a Perkin Elmer Applied Biosystems Model 494 Procise protein/peptide sequencer with an on-line Perkin Elmer Applied Biosystems Model 140C PTH Amino Acid Analyzer. The chemical process deployed by the protein sequencer to determine the amino acid sequence was derived from the Edman degradation (Edman *et al*. 1950).The sterile dried PVDF membrane having protein band of interest was used for N-terminal sequencing.

3.6. Molecular Characterization of Anti-*Candida* Protein (ACP)

3.6.1. Locus identification of ACP Genes (Plasmid Curing)

Plasmid curing was done to check the location of antimycotic genes whether these genes are in plasmid or genomic DNA, with different concentrations of novobiocin according to the method followed by Ruiz-Barba (1991). Overnight grown culture of *E. faecalis* was inoculated in TS broth containing 1 µg/ml novobiocin and grown for 72 h at 15°C and 37°C, then subcultured by increasing the concentration of novobiocin up to the MIC of the ACP.

Finally at 60 µg/ml novobiocin concentration, the culture was passaged in presence of ascorbic acid and $CuCl_2$ solution at a final concentration of 1 mM and 20 µM respectively for 35 generations. Then the culture was serially diluted to 10^{-13} dilution and spread on TS agar plates. The colonies were replica plated on to TS agar plates with 100 µg/ ml kanamycin by tooth pick method. The colonies those were not observed in the replica plate were picked from the master plate and cultured for overnight and inoculated in to lysis broth. The residual antimicrobial activity of cell free culture supernatant was checked. The plasmid isolation was carried out using modified Anderson and Mckay (1983), method and the electrophoresis was done using 0.8% agarose gel to the presence of plasmid.

3.6.2. Genomic DNA Extraction and Quantification

DNA extraction was carried out twice via the Pospiech and Neumann (1995) method. Overnight grown cells of the test strain in TSB (30 ml), pH 7.2, were harvested by centrifugation (10000 rpm for 10 minutes) and resuspended in 5 ml of SET (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5), 100 µl of lysozyme (1.0 mg/ml) was added. The tube was incubated at 37°C for 60 minutes. Then 1/10 volumes of 10% SDS was added followed by 0.5mg/ml proteinase K (10 mg/ml stock solution) and incubated at 55°C with occasional inversion of 90 minutes.1/3 volumes of 5.0 M NaCl and 1 volume of chloroform was added and incubated at room temperature for 30 minutes with frequent inversion, centrifuged at 6000 rpm for 15 min and transferred the aqueous phase to a new tube using blunt-ended sterile tip.

Phenol/chloroform/isoamyl alcohol (PCI) extraction was done. RNase was added to the aqueous phase at the rate of 10µg/ml and incubated at 37°C for 40 minutes. Again PCI extraction was done. Upper aqueous layer was collected using sterile blunt-ended tips and transferred to a new sterile microfuge tube. DNA was precipitated by adding 1 volume of isopropanol or 2.5 volume of absolute alcohol, and stored the tube at -20°C after mixing. DNA was transferred into microfuge tube, centrifuged and rinsed with 1 ml of 70% alcohol; air dried the DNA pellet and dissolved in a suitable volume of nuclease free TE buffer (10:0.1), pH 8.0. DNA was stored at -20°C and an aliquot of 5.0 µl was run on 0.7 % agarose gel.

3.6.3. Genomic DNA Library Construction

Quantization of Genomic DNA

The DNA was diluted 15 times while estimation = 20 µl DNA + 280 µl TE.

Extraction 1

O.D at 260 nm	= 1.030
O.D at 280 nm	= 0.475
O.D at 260 / O.D at 280	= 2.16
Concentration of DNA	= 1.030 × 15 × 50= 772.5 µg/ ml

Extraction 2

O.D. at 260 nm	= 1.040
O.D. at 280 nm	= 0.472
O.D. at 260 / O.D at 280	= 2.20
Concentration of DNA	= 780 µg/ ml

Small Scale Partial Genomic DNA Digestion

1) DNA	= 25 µg = 33 µl
*Sau*3AI buffer (10X) (Promega)	= 30 ul (3ul BSA (1mg/ ml) + 27µl Multi core buffer
Restriction Enzyme (Promega)	= 1ul (0.5 U/ µl)
SDW	= 236 µl

Total	= 300µl
2) DNA	= 25 µg = 33 µl
*Sau*3AI buffer (10X)	= 30ul (3ul BSA (1mg/ ml) +27µl Multi core buffer)
RE	= 1ul (0.25 U/ µl)
SDW	= 236 µl
Total	= 300µl

Both reactions were set in a water bath at 37°C for 1 hr. After analyzing the band in the form of smear obtained the large scale digestion was done with 0.0625 U/ µg of DNA.

Large Scale Partial Genomic DNA Digestion

1) Genomic DNA	= 50 µg = 65µl
*Sau*3AI Buffer (10X)	= 60 µl (6µl BSA (1mg/ml) + 54µl Multi core buffer)
SDW	= 471.8µl
RE	= 3.2µl (1Uµl)
Total	= 600 µl

The reaction was set in a water bath at 37°C for 1 hr.

The tubes were incubated at 37 °C for 65 minutes. $1/10^{th}$ volume of 3M sodium acetate, pH 5.2 and $1/10^{th}$ volume of 0.5 M EDTA was added to each tube and then phenol/chloroform/isoamyl alcohol (PCI, 25:24:1) extraction was performed. Then, 3X volumes of absolute alcohol were added and stored at -20°C overnight. The DNA was then centrifuged (10,000 rpm for 10 min at 4°C), precipitated and re-suspended in 40 µl TE (10:1) per tube.

Small Scale Complete Digestion of pUC19 (GeNeI)

pUC19	= 1µg = 10 µl (100µg/ ml)
*Bam*HI (10X) buffer	= 2 µl
*Bam*HI (Promega)	= 2 µl
SDW	= 6 µl
Total	= 20 µl

Mixed and incubated at 37 °C for 90 minutes.

Large Scale Complete Digestion of pUC19

pUC19	= 25 µg = 250 ul (100ng/ µl)
*Bam*H1 (10X) Buffer	= 50 µl
*Bam*H1	=15 µl (10U/ µl)
SDW	=185 µl
Total	= 500 µl

Mixed and incubated at 37°C for 3 hours.

After large scale digest, $1/10^{th}$ volume of 0.5 M EDTA pH 8.0 was added and again PCI extraction was done. Then 3X volume of chilled absolute alcohol was added and the tubes were stored in -20°C. The DNA was then centrifuged (12000 rpm for 10 min at 4°C), precipitated and re-suspended in 40 µl TE (10:1) per tube. The two tubes were then pooled together.

Dephosphorylation of BamH1 Digested pUC19 vector

Vector DNA	= 40 µl (20µg)
10X CIAP buffer	= 10 µl
CIAP (2U/ul) (Fermentas)	= 1 µl
SDW	= 49 µl
Total	= 100 µl

The reaction mix was incubated in a water bath at 37°C for 30 minutes, then 56°C for 15 min. 1 µl of calf intestine alkaline phosphatase (CIAP) was added for 30 minutes and heated the tubes at 70°C for 15 minutes. Then PCI extraction was done and finally the pellet obtained was dissolved after alcohol precipitation in 100 µl of nuclease free sterile distilled water and stored at -20°C until use.

Quantization of partially Digested DNA

The partially digested DNA was diluted 35 times for the purpose of quantization.

10ul of DNA was diluted to 350 ul using TE buffer (10:1).

O.D at 260 nm	= 0.520
O.D at 280 nm	= 0.255
Concentration	= 910 µg/ ml
O.D at 260/ O.D at 280	= 2.039

Ligation of vector and insert

The following reactions were setup

Vector: Insert = 2:1

Dephosphorylated vector DNA/ Insert DNA= 1µl insert (diluted to 200 ng/µl with SDW) + 4µl plasmid (100ng/µl)

SDW	= 2 µl
10 X ligation buffer	= 1 µl
T4 DNA ligase	= 2 µl
Total	= 10 µl

Vector: Insert = 1:2

Dephosphorylated vector DNA/ Insert DNA = 2 µl insert (diluted to 200ng/µl with SDW) +2µl plasmid (100 ng/µl)

SDW	= 3 µl
10X ligation buffer	= 1 µl
T4 DNA ligase (Promega)	= 2 µl
Total	= 10 µl

The ligation mix was incubated at 16°C for 12 hours.

Cloning and transformation of genes in *E. coli*

Preparation of competent cells (*E. coli* DH5α) and transformation was carried out for both the ligation mixes. Transformed cells were plated on AIX plates, Ampicillin 100 µg /ml, 35 µl IPTG (20 mg/ml) as gratuitous inducer and 35 µl of X-Gal (20 mg/ml) as chromogenic substrate (Sambrooke standard protocol).

3.6.4. Induction Studies of ACP Gene using Mitomycin C

A 24-h culture was used to inoculate mTSB at the rate of 4% (10^8 CFU/ml). Mitomycin C was added at a concentration of 2.0, 5.0 and 10 µg ml^{-1} in the culture tubes (Echandi and Moyer 1979) and a tube without mitomycin C was used as control. The tubes were pre-incubated for 30 and 60 min with mitomycin C at 14°C. Following the incubation, cells were harvested by centrifugation at (12000 rpm for 30 min at 4°C).

The cells were then, resuspended in fresh mTS broths and incubated for 30 hours at 14ºC. At every 4 hrs interval (till 30 h), 1 ml of the culture was centrifuged at 12000 rpm at 4ºC /30 min to collect the CFS and stored at -80ºC until use. Simultaneously the absorbance of the culture was also measured at 600 nm. The antimicrobial activity of the cell free supernatant (CFS) was tested against *C. albicans* MTCC 3958 (Shekh *et al*. 2011; Shekh and Roy 2012). The experiment was done in duplicate.

3.7. Therapeutic Approach of ACP

3.7.1. Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the dialyzed concentrate against *C. albicans* (MTCC 183, MTCC 3958, MTCC 7315 and wild type *C. albicans* DI) was determined by the microbroth dilution assay in a 96-well microtitre plate (Tarsons). *C. albicans* (10^6 CFU/ ml) was checked for sensitivity to 2-fold increasing dilutions of the compounds (2.165 to 0.00099 mg/ml). Turbidity was determined to monitor cell growth (Hasan *et al*. 2009) after incubation at 37 °C for 36 h. The MIC was defined as the lowest concentration of the compounds inhibiting the yeast growth.

3.7.2. Study of Hemolytic Activity Against Human Erythrocytes

The hemolytic activity of the antifungal dialyzed concentrate on human erythrocytes was determined based on Yadav *et al*. (2005). Human erythrocytes in 2% (v/v) suspension were exposed to various concentrations of ACP ranging from 6.4 to 0.00156 mg/ml at 37 ˚C for 1 h. The cells were peletted at 600 rpm for 10 min and the supernatant was collected to determine the absorbance at 450 nm using a UV-Visible Spectrophotometer (Shimadzu). Erythrocyte suspension and phosphate buffered saline (PBS) was used as negative control sets whereas in positive controls, lysis buffer was used for completely lysing the erythrocytes. The percentage hemolysis was calculated and plotted against the concentration of ACP to determine the dose cytotoxic to human erythrocytes. The percentage of intact erythrocytes was calculated using the following formula.

$$\text{Percent of intact erythrocytes} = \left[1 - \frac{\text{Absorbance of protein - Absorbance of PBS}}{\text{Absorbance of lysis buffer-Absorbance of PBS}} \right] \times 100$$

$$\text{Percent of hemolysis} = 100 - (\text{Percent of intact erythrocytes})$$

3.7.3. Haemagglutination Activity Assay against Human RBC

This assay was conducted to determine whether *E. faecalis* exhibits haemagglutination activity based on Wong *et al*. (2008). In a microtitre U-plate, 50 µl of PBS alone was added in the first well (control) and in the other wells, serial two fold dilution of the dialysed concentrate (50 µl) was carried out using PBS (concentration of the protein used 6.4 mg/ml- 0.001mg/ml). Then, 50 µl of RBC suspension was mixed with the sample in all the wells**.** The plate was incubated at 20ºC for 1 hour and then the plate was visually inspected for haemagglutination (Wong *et al.* 2008).

CHAPTER 4

RESULTS

Chapter 4 *RESULTS*

4.1. Isolation and Characterization of Antimicrobial Substance (AMP) producing Strains

4.1.1. Isolation and Growth Characteristics

As many as 240 isolates from different areas of the Arctic and Antarctic (Table 5), were collected from the National Centre for Arctic and Ocean Research (NCAOR) Sada Headland, Goa. These isolates grew well at 0 to 5.0% NaCl and one Antarctic Penguin Rookery isolate (APR 210) was tolerant up to 6.5% NaCl, but showed no growth at 9.0% NaCl. The selected strains had no visible growth at 0°C, moderate growth at 5°C, and good growth at 10–22°C.The selected strains grew well in pH 7.0–9.0. The Arctic sea ice bacterial community consists mostly of psychrotrophic and halotolerant species. Penguin rookery bacteria are psychrophilic and produce antimicrobial compounds (AMPs) (Shekh *et al*. 2009).

Table 5. Sampling sites of biologically active strains (a) GMWSC, glacial melt water and sea convergence. (b) APR, Antarctic penguin rookery; AGM, Arctic glacial melt.

Area	**Source[a]**	**Strain code[b]**
Larsemann Hills	Penguin rookery	APR 12
Larsemann Hills	Penguin rookery	APR 79
Larsemann Hills	Penguin rookery	APR210
Schirmacher Oasis	Priyadarshini Lake	4A
Schirmacher Oasis	Priyadarshini Lake	21C
Arctic sea	GMWSC	AGM108-5
Arctic sea	GMWSC	AGM111

4.1.2. Test for Antimicrobial Activity

All the collected strains were checked for the production of antimicrobial factors against different indicator organisms (Table 6). Among these strains, seven strains showed antimicrobial activity against yeast as well as bacterial pathogens. One most promising isolate named APR210 showed broad-spectrum biological activity against different multidrug resistant yeast pathogens as well as wild bacterial indicator organisms (Table 7).

Table 6. Different indicator organisms purchased from the MTCC, Chandigarh and the NCIM, Pune, India, and other wild-type indicator organisms used in the antimicrobial activity assay against APR210. SC 5314* was obtained from JNU, New Delhi.

Strain number	**Organisms**
NCIM 3471	*Candida albicans*
MTCC 183	*C.albicans*
MTCC 3958	*C.albicans*
NCIM 3557	*C.albicans*
MTCC 227	*C.albicans*
MTCC 7315	*C.albicans*
Wild-type (Goa DI)	*C.albicans*
Wild type (Dehradun WI)	*C.albicans*
SC 5314*	*C.albicans*
NCIM 3129	*C.krusei*
NCIM 3541	*C.neoformans*
NCIM 857	*Aspergillus flavus*
------	*A. niger*
------	*Ps.aeruginosa*
MTCC 2080	*E. faecalis*
------	*Ps. putida*
Wild type	*Bacillus sp.*
Wild type (108-5)	*Yersinia pestis*

Table 7. Antimicrobial activity of selected producer strains against various *Candida* strains.

Strain Code	Antimycotic activities zones on *Candida* spp (mm)			
	NCIM 3471	MTCC 7315	MTCC 183	MTCC 3129
APR-12	17	-	-	10
APR-79	15	-	-	10
APR210	20	23	23	10
4A	22	13	12	-
21C	12	-	-	-
AGM108-5	20	15	15	12
AGM111	18	15	18	11

4.1.3. Morphological and Biochemical Characterization

Most of the selected strains were Gram-positive bacilli and cocci. Most of the selected strains such as APR210, AGM108-5, and AGM111 were non-motile, acid-producing, catalase-negative, and grew in MRS medium. AGM108-5 and AGM111 were oxidase-positive (Table 8). Strain AMG111 was non-spore-forming, with cells occurring as straight rods (singly, as pairs, or as short chains) (Table 8). APR210 was Gram-positive, catalase-negative, oxidase-negative, facultative anaerobic, and heterofermentative (Table 9). It fermented sugars, namely glucose, lactose, and sucrose, and arabinose to some extent (Table 9). It grew well at 10°C, 15°C, and 22°C, as well as in 2–5% and upto 6.5% NaCl, but not in 9% NaCl (Table 8).

Amongst the 240 isolates tested, only 7 isolates showed inhibition against the selected *C. albicans* (NCIM 3471). Out of these, 5 were able to grow in mTSB medium containing 5% (w/v) NaCl. However, most of the strains also grew in medium lacking NaCl; therefore, they are halotolerant, not halophilic. The selected strains had a chemoorganoheterotrophic metabolism and were capable of growth on the following substrates; D-arabinose, raffinose, ribose, D-glucose, D-fructose, D-mannose, D-maltose, sucrose, lactose, and sorbitol. All the isolates were capable of fermenting glucose (Table 8). The best growth was observed in D-glucose, sucrose and the weakest growth was there in D-arabinose (Table 8), containing mTSB medium.

Since all of the isolates experience constant cold in their natural habitat, their cardinal temperatures were a major focus of our study; the results are shown in Table 8. Apart from this, APR210 was isolated from faeces and feather samples of penguins, and gray-white, smooth colonies of diameter 1–5 mm were recovered from MGYP agar plate incubated at 5°C for 2 weeks. The strain was able to grow at 10°C and 37°C in presence of 6.5% NaCl but failed to grow at 10 and 45°C with 6.5% salt. However, it was able to grow at 10 and 45°C in absence of NaCl and at pH 9.6. The strain was unable to produce gas from glucose fermentation at 22º, 25º, and 37°C and was found to ferment arabinose weakly.

Minimal growth temperatures for the isolates ranged from 4°C to 22°C. The optimum temperature for most of the cultures in the present study was observed to be 15–18°C. The two isolates 210 and 211 grew in temperatures as low as 10°C, with the optimum near 18°C. However, the maximum growth temperatures were as high as 37°C. Interestingly, no strong correlation was observed between the enrichment temperature and growth temperature limits, as isolates with any enrichment temperature grew at temperatures as high as 37 °C. Since the organisms were isolated from penguin rookery samples, they must have grown at comparatively higher temperatures, and adapted to grow at 37°C. These results also indicate that psychrophily may not be common in prokaryotes from this permanently cold environment.

The biochemical profile of the most promising isolate, APR 210, was determined based on the colour change in the BK009 HiCarbohydrate kit (Figure 2). APR 210 fermented mannitol, glucose, lactose, sucrose, sorbitol, and arabinose and it tested positive for esculin hydrolysis (Table 10). Andrew (1900) viewed mannitol fermentation as one of the criteria for the classification of *Enterococci* (Sherman *et al*. 1937). According to Meyer and Schonfeld, the bile esculin test shown by Facklam and Moody was sensitive (100%) and specific (97%) in identifying group D *Streptococci* and *Enterococci* (Chuard *et al*., 1988).

Table 8. Morphological and biochemical characteristics of selected AMP-producing isolates

Characteristics	**AGM(108-5)**	**APR210**	**AGM111**
Morphology	Bacilli	Cocci	Bacilli
Gram studies	Gram -ve	Gram +ve	Gram +ve
Acid production	+	+	+
Gas production	+	-	-
Motility	-	-	-
Growth in diff. conc. of NaCl			
0%	+	+	+
2.0%	+	+	+
5.0%	+	+	+
6.5%	-	+	-
9.0%	-	-	-
Growth in diff. temp.			
0ºC	-	-	-
5 ºC	+	-	+
15 ºC	+	+	+
18 ºC	+	+	+
22 ºC	+	+	+
Catalase test	-	-	-
Oxidase test	+	-	+
Growth in pH 7.0- 9.0	+	+	+
Growth in MRS	+	+	+

Table 9. Acid production of isolated strains in different sugars

Acid production	**12 APR**	**APR-79**	**AGM (108-5)**	**APR 210**	**AGM 111**	**4A**	**21C**
D-Arabinose	+-	+-	+	+-	+-	+-	+-
D-Ribose	+	+	+	+	+	+	+
Raffinose	+	+	-	+	+	+	+
Glucose	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+
Lactose	+	+	-	+	+	+	+
Mannose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+

Figure 2. Biochemical profile study of *E. faecalis* APR210 based on the BK009 HiCarbohydrate kit.

Table 10. Biochemical profile study of *E. faecalis* APR 210 based on BK009 HiCarbohydrate kit.

Sugars & Reagents	**Results**	**Sugars & Reagents**	**Results**	**Sugars & Reagents**	**Results**
Lactose	+	Inulin	+	Rhamnose	-
Xylose	-	Sodium gluconate	-	Cellobiose	+
Maltose	+	Glycerol	+	Melezitol	+
Fructose	+	Salicin	+	α-Methyl D-Mannoside	+
Dextrose	+	Dulcitol	-	Xylitol	-
Galactose	+	Inocitol	-	ONPG	-
Raffinose	+	Sorbitol	-	Esculin hydrolysis	+
Trehalose	+	Mannitol	+	D-Arabinose	+
Malibiose	+	Adonitol	-	Citrate utilization	-
Sucrose	+	Arabitol	-	Malonate utilization	+
L-Arabinose	+	Erythritol	-	Sorbose	-
Mannose	+	α-Methyl D-Glucoside	+		

4.1.4. Fatty Acid Methyl Ester Parameters of AMP Producing Strains

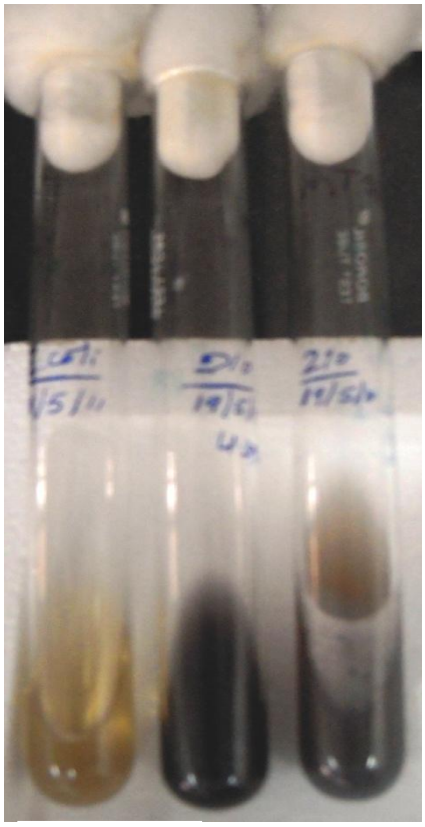
The fatty acid compositions of the strains APR210, APR111, AGM108-5, and AGM111 are given in Table 11. Based on fatty acid composition, AGM108-5 was similar to *Enterobacter hormaechei* (similarity index, 0.813), showing an abundance of unsaturated fatty acids like vaccenic, palmitic, and palmitoleic acids (approximately 22%). The organism also contained petroselinic acid (18:1 ω6c), one of the major fatty acids. AGM111 was 93% similar to *Brevibacterium incertum*. The other 2 isolates, APR210 and APR211, were 99.4 and 99.6% related, respectively, to an *E. faecalis* strain (ATCC 828); therefore, it is probable that these 2 isolates are of the same species and are identical. Thus, identification of the isolates was achieved; based on the morphological and biochemical characteristics, and the FAME test, the isolate APR210 was confirmed to be *E. faecalis.*

Table 11. Fatty acid composition of the selected strains. (-) indicates that the fatty acid was not determined.

Compounds	**AGM(108-5)**	**AGM111**	**APR 210**	**APR 211**
10:0 FAME	-	-	-	-
12:0 FAME	4.66	-	-	-
14:0 FAME	5.39	14.01	10.84	5.77
Summed feature 1	1.14	-	-	-
Summed feature 2	8.71	-	-	-
Summed feature 3	21.22	2.53	17.5	15.91
16:0 FAME	23.37	17.72	22.55	15.65
17:0 FAME	1.42	-	-	-
Summed feature 8	22.02	-	28.47	39.47
18:0 FAME	-	-	-	-
12:0 3OH	-	-	-	-
16:1 ω9c	-	13.69	-	-
17:0 cyclo	8.83	-	1.31	-
17:0 iso	-	-	-	-
17:1 ω8c	-	-	-	-
18:1 ω9c	-	43.53	-	-
19:0 cyclo ω8c	-	-	17.38	20.91
20:1 ω9c	-	2.23	-	-

4.1.5. Potassium Tellurite Reduction by APR210

The primary purpose of the tellurite tolerance test was to aid in the differentiation of *E. faecalis*, *E. faecium*, and other *Enterococci* (Murray *et al*. 2003). Tolerance (a positive result) was indicated whenever black colonies form on the surface or in broth. Typical and variant strains of *E. faecalis* usually form black coloration (positive tolerance) after 48 h of incubation (Figure 3). Some strains of *E. faecium* may form grey colonies (a negative reaction), but most stains fail to grow in tellurite medium. A slight blackening at the bottom of the slant is a negative result. Here, it was determined that APR210 is indeed *E. faecalis*, confirming the results of the FAME analysis.



E.coli **APR210**

Figure 3. Black coloration suggested the reduction of tellurite, confirming the identity of APR210 as *E. faecalis*. *E. coli* was used as a negative control.

4.1.6. 16S rDNA Sequencing and Identification of Selected Bacterial Strains

The identification of the selected isolates was confirmed by 16 S rDNA sequence analysis. The sequence obtained (Figure 4), was compared with all sequences presently available in the GenBank database (NCBI), and the isolate AGM111 appeared to be highly similar to sequences from *Carnobacterium* species. According to the pairwise distance table (not shown), strain AGM111 appears to belong to a cluster including *C. maltaromaticum, C. piscicola* and *Lactobacillus maltaromaticus* with 99.6 (GenBank accession number AM179873), 99.9 and 86.8 similarity, respectively (Shekh *et al*. 2011).

16S rDNA phylogenetic studies confirmed that strain AGM108-5 belongs to the genus *Yersinia*. The similarities shown by AGM108-5 to *Yersinia enterocolitica* and *Y. intermedia* were 100% and 94% respectively.

The 16S rDNA sequence of the isolate APR210 showed the closest homology (99.0%) to an *E. faecalis* strain (Accession No. GU39800.1), according to the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST), whereas according to EzTaxon server version 2.1, the APR210 sequence showed the closest homology (99.3%) to an *E. faecium* strain (Accession No.DQ411813). FAME analysis suggested, however, that APR210 was most closely related to the *E. faecalis* strain ATCC 828. The *E. faecalis* and *E. faecium* species were also examined based on the reduction of potassium tellurite, the results of which strongly suggested that the *E. faecalis* species was most closely related.

E. faecalis (APR210)

GCTCTTTTTAACGGAGCTTGCTCACCGGAAAGAAGAGTGGCGAACGGGTGAGTAACACGTGG
GTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAATCG
AAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGC
ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGT
GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATC
TTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCG
TAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGTTCATCCCTTGACGGTATCTA
ACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTG
TCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCG
GCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATT
CCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCT
GGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTA
ACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGG
GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGG
TCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGGTGG
TGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTATTGTTAGTTGCCATCATTCAGTTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAGG
AAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATG
GGAAGTACAACGAGTTGCGAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCG
GATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGC
CGCGTGACA

Figure 4. The sequence of the biologically active strain (APR210), obtained by 16S rDNA gene sequencing.

4.1.7. Phylogenetic Tree of Selected Strain (APR210)

The phylogenetic dendrogram of APR210 is shown in Figure 5. The nearest homolog of the strain based on NCBI BLAST search is shown in the dendrogram (Figure 5). This strain showed the most similarity to *Enterococcus faecalis*, the presence of *E. faecalis* corroborates earlier findings regarding the presence of the Gram-positive bacterium *S. faecalis* in penguins (Soucek *et al*. 1970).

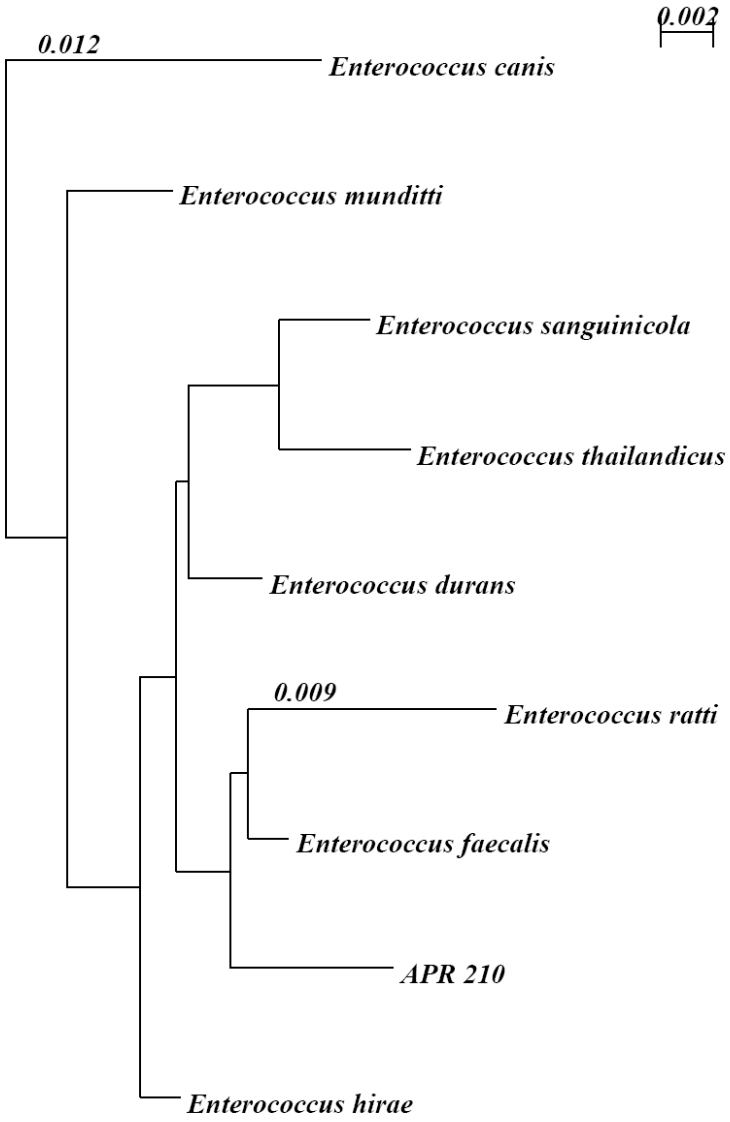


Figure 5. Phylogenetic tree generated using Clustal X software using the neighbour-joining method (NJ-Plot), showing the divergence of the isolated strain APR210 from ten of its closest homologs as identified by NCBI BLAST.

4.1.8. Antibiotic Resistance in Selected Isolates

The sensitivity of three selected biologically active strains against several antibiotics are listed in Table 12. AGM108-5 was sensitive to ampicillin, ampicilin/ cloxacillin, chlortetracycline, chloramphenicol, streptomycin, kanamycin, and neomycin and resistant to tetracycline, rifampicin, vancomycin, and novobiocin. The selected isolate APR210 was sensitive to ampicillin, ampicillin/cloxacilln, tetracycline, chlortetracycline, chloramphenicol, rifampicin, and novobiocin, and resistant to streptomycin vancomycin, kanamycin and neomycin.

Table 12. Sensitivity and resistance of selected biologically active strains against several antibiotics. S indicates sensitivity, R indicates resistance.

Characteristics	**Sensitivity of selected Strains**		
Antibiotics (mcg/disc)	**AGM(108-5)**	**APR210**	**AGM111**
Ampicillin (10)	S	S	S
Ampicillin / Cloxacilln (10)	S	S	S
Tetracyclin (10)	R	S	R
Chlortetracyclin (20)	S	S	S
Chloramphenicol (30)	S	S	S
Streptomycin (25)	S	R	S
Rifampicin (10)	R	S	S
Vancomycin (10)	R	R	S
Novobiocin (30)	R	S	R
Kanamycin (30)	S	R	S
Neomycin (30)	S	R	S

4.2. Characteristics of Indicator Organisms

4.2.1. Growth Parameters

Most of the yeast indicator organisms (*C. albicans* and *C. crusei*) were purchased from MTCC Chandigarh and NCIM Pune in India, and grown based on their guidelines. Most of the *C. albicans* strains (MTCC 183, MTCC 3958, and MTCC 3471) grew well at room temperature (25–30°C), whereas some wild-type *C. albicans* strains (DI and WI) grew well at 37°C in MGYP medium (pH 6.2 ± 0.4).

4.2.2. Identification of Wild-type Indicator Organisms

The wild-type indicator organism DI was found to be closest *C. albicans* (NCBI Acc No. M60302.1|YSASRSUA; the identified sequence is given in Figure 6a, whereas the wild-type strain WI was found to be closest to *C. albicans* (NCBI Acc No. AJ005123) with score 99%. The sequence of WI is given in Figure 6b. The phylogenetic tree of the DI and WI indicator organisms is shown in Figure 7.

GATGACAGCTTCTCGGTTCAGAATGAGGTTGCCCCCTTTCCTAAACCAATCCGGAGGCCTCACTAAGCCA
TTCAATCGGTAGTAGCGACGGGCGGTGTGTACAAAGGGCAGGGACGTAATCAACGCAAGCTGATGACTT
GCGCTTACTAGGAATTCCTCGTTGAAGAGCAACAATTACAATGCTCTATCCCCAGCACGACGGAGTTTCA
CAAGATTTCCCAGACCTCTCGGCCAAGGCTTATACTCGCTGGCTCCGTCAGTGTAGCGCGCGTGCGGCCC
AGAACGTCTAAGGGCATCACAGACCTGTTATTGCCTCAAACTTCCATCGACTTGAAATCGATAGTCCCTC
TAAGAAGTGACTATACCAGCAAATGCTAGCAGCACTATTTAGTAGGTTAAGGTCTCGTTCGTTATCGCAA
TTAAGCAGACAAATCACTCCACCAACTAAGAACGGCCATGCACCACCACCCACAAAATCAAGAAAGAGC
TCTCAATCTGTCAATCCTTATTGTGTCTGGACCTGGTGAGTTTCCCCGTGTTGAGTCAAATTAAGCCGCAG
GCTCCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCGACCATACTCCCCCCAGAA
CCCAAAGACTTTGATTTCTCGTAAGGTGCCGATTGCGTCAATAAAAGAACAACAACCGATCCCTAGTCGG
CATAGTTTATGGTTAAGACTACGACGGTATCTGATCATCTTCGATCCCCTAACTTTCGTTCTTGATTAATG
AAAACGTCCTTGGTAAATGCTTTCGCAGTAGTTAGTCTTCAGTAAATCCAAGAATTTCACCTCTGACAACT
GAATACTGATACCCCCGACCGTCCCTATTAATCATTACGATGGTCCTAGAAACCAACAAAATAGAACCAT
AACGTCCTATTCTATTATTCCATGCTAATATATTCGAGCAAAGGCCTGCTTTGAACACTCTAATTTTTTCA
AAGTAAAAGTCCTGGTTCGCCATAAATGGCTACCCAGAAGGAAAGGCTCGGCTGGGTCCAGTACGCATC
AAAAAGATGGACCGGCCAGCCAAGCCCAAGGTTCAACTACGAGCTTTTTAACTGCAACAACTTTAATATA
CGCTTTTGGAGCTGGAATTACCGCGGCTGCTGGCACCAGACTTGCCCTCCAATTGTTCCTCGTTAAGGTAT
TTACATTGTACTCATTCCAATTACAAGACCCAAAAGGGCCCTGTATCGTTATTTATTGTCACTACCTCCCC
GTGTCGGGATTGGGTAATTTGCGCGCCTGCTGCCTTCCTTGGATGTGGTAGCCGTTTCTCAGGCTCCCTCT
CCGGAATCGAACCCTTATTCCCCGTTACCCGTTGAAACCATGGTAGGCCACTATCCTACCATCGAAAGTT
GATAGGGCAGAAATTTGAATGAACCATCGCCAGCACAAGGCCATGCGATTCGAAAAGTTATTATGAATC
ATCAAAGAGCCCGAAGGCATTGATTTTTTATCTAATAAATACATCCCTTCCAAACAGTCGGGATTTTAAG
CATGTATTAGCTCTAGAATTACCACGGTTATCCAAGTAGTAAGGTACTATCAAATAAACGATAACTGATT
TAATGAGCCATTCGCAGTTTCACTGTATAAATTGCTTATACTTAGACATGCATGGCTAATCTAGGACTG

Figure 6 a. Sequence (1683 bp) of wild-type *C. albicans* (DI) isolated from a diabetic patient.

ATTTGACAGCTTCTCGGTTCCAGAATGAGGTTGCCCCCTTTCCTAAACCAATCCGGAGGCCTCACTAAGC
CATTCAATCGGTAGTAGCGACGGGCGGTGTGTACAAAGGGCAGGGACGTAATCAACGCAAGCTGATGAC
TTGCGCTTACTAGGAATTCCTCGTTGAAGAGCAACAATTACAATGCTCTATCCCCAGCACGACGGAGTTT
CACAAGATTTCCCAGACCTCTCGGCCAAGGCTTATACTCGCTGGCTCCGTCAGTGTAGCGCGCGTGCGGC
CCAGAACGTCTAAGGGCATCACAGACCTGTTATTGCCTCAAACTTCCATCGACTTGAAGTCGATAGTCCC
TCTAAGAAGTGACTATACCAGCAAATGCTAGCAGCACTATTTAGTAGGTTAAGGTCTCGTTCGTTATCGC
AATTAAGCAGACAAATCACTCCACCAACTAAGAACGGCCATGCACCACCACCCACAAAATCAAGAAAGA
GCTCTCAATCTGTCAATCCTTATTGTGTCTGGACCTGGTGAGTTTCCCCGTGTTGAGTCAAATTAAGCCGC
AGGCTCCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCGACCATACTCCCCCCAG
AACCCAAAGACTTTGATTTCTCGTAAGGTGCCGATTGCGTCAATAAAAGAACAACAACCGATCCCTAGTC
GGCATAGTTTATGGTTAAGACTACGACGGTATCTGATCATCTTCGATCCCCTAACTTTCGTTCTTGATTAA
TGAAAACGTCCTTGGTAAATGCTTTCGCAGTAGTTAGTCTTCAGTAAATCCAAGAATTTCACCTCTGACAA
CTGAATACTGATACCCCCGACCGTCCCTATTAATCATTACGATGGTCCTAGAAACCAACAAAATAGAACC
ATAACGTCCTATTCTATTATTCCATGCTAATATATTCGAGCAAAGGCCTGCTTTGAACACTCTAATTTTTTC
AAAGTAAAAGTCCTGGTTCGCCATAAATGGCTACCCAGAAGGAAAGGCTCGGCTGGGTCCAGTACGCAT
CAAAAAGATGGACCGGCCAGCCAAGCCCAAGGTTCAACTACGAGCTTTTTAACTGCAACAACTTTAATAT
ACGCTTTTGGAGCTGGAATTACCGCGGCTGCTGGCACCAGACTTGCCCTCCAATTGTTCCTCGTTAAGGTA
TTTACATTGTACTCATTCCAATTACAAGACCCAAAAGGGCCCTGTATCGTTATTTATTGTCACTACCTCCC
CGTGTCGGGATTGGGTAATTTGCGCGCCTGCTGCCTTCCTTGGATGTGGTAGCCGTTTCTCAGGCTCCCTC
TCCGGAATCGAACCCTTATTCCCCGTTACCCGTTGAAACCATGGTAGGCCACTATCCTACCATCGAAAGT
TGATAGGGCAGAAATTTGAATGAACCATCGCCAGCACAAGGCCATGCGATTCGAAAAGTTATTATGAAT
CATCAAAGAGCCCGAAGGCATTGATTTTTTATCTAATAAATACATCCCTTCCAAACAGTCGGGATTTTAA
GCATGTATTAGCTCTAGAATTACCACGGTTATCCAAGTAGTAAGGTACTATCAAATAAACGATAACTGAT
TTAATGAGCCATTCGCAGTTTCACTGTATAAATTGCTTATACTTAGACATGCATGGCTAATCTAAGATG

Figure 6 b. Sequence (1684 bp) of wild-type *C. albicans* (WI) from Dehradun.

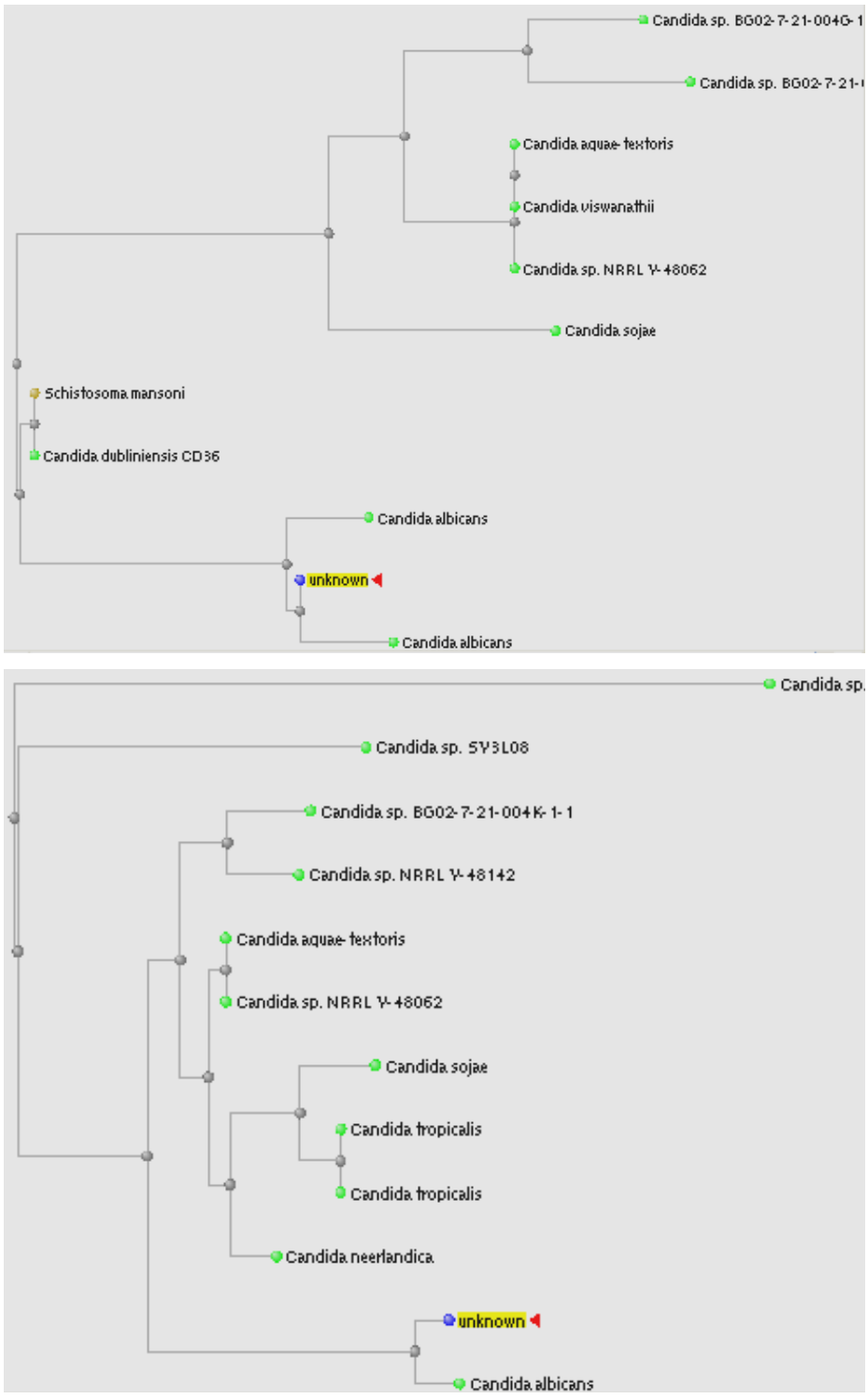


Figure 7. Phylogenetic trees of DI and WI indicator organisms showing their nearest *C. albicans* homologues.

4.2.3. Antifungal Susceptibility Testing of Indicator Organisms

The results of antifungal susceptibility testing of different indicator organisms are given in Table13. Most of the *C. albicans* strains (MTCC 183, MTCC 3958, MTCC 3471, MTCC 7315, and wild-type DI and WI) and SC 5314 were tested (Figure 8), and found to be resistant to azoles as well as polyenes, i.e. amphotericin B and other antifungal drugs. Most of the indicator organisms, with the exception of the wild-type WI, were sensitive to the antifungal drug voriconazole.

Table 13. Susceptibility of various *C. albicans* indicator strains to antifungal antibiotics.

Antifungal Abts (mcg/disc)	**Different *C. albicans* (Indicator organisms)**						
	MTCC 183	**MTCC 3958**	**NCIM 3471**	**MTCC 7315**	**WI**	**DI**	**SC 5314**
Clotrimazole	R	R	R	R	R	R	R
Miconazole	R	R	R	S	R	R	R
AmphotericinB (20)	R	R	S	R	R	R	S
Nystatin	R	R	R	R	S	R	R
Fluconazole	R	S	S	R	R	S	R
Voriconazole	S	S	S	S	R	S	S
AmphotericinB (10)	R	R	R	R	R	R	R

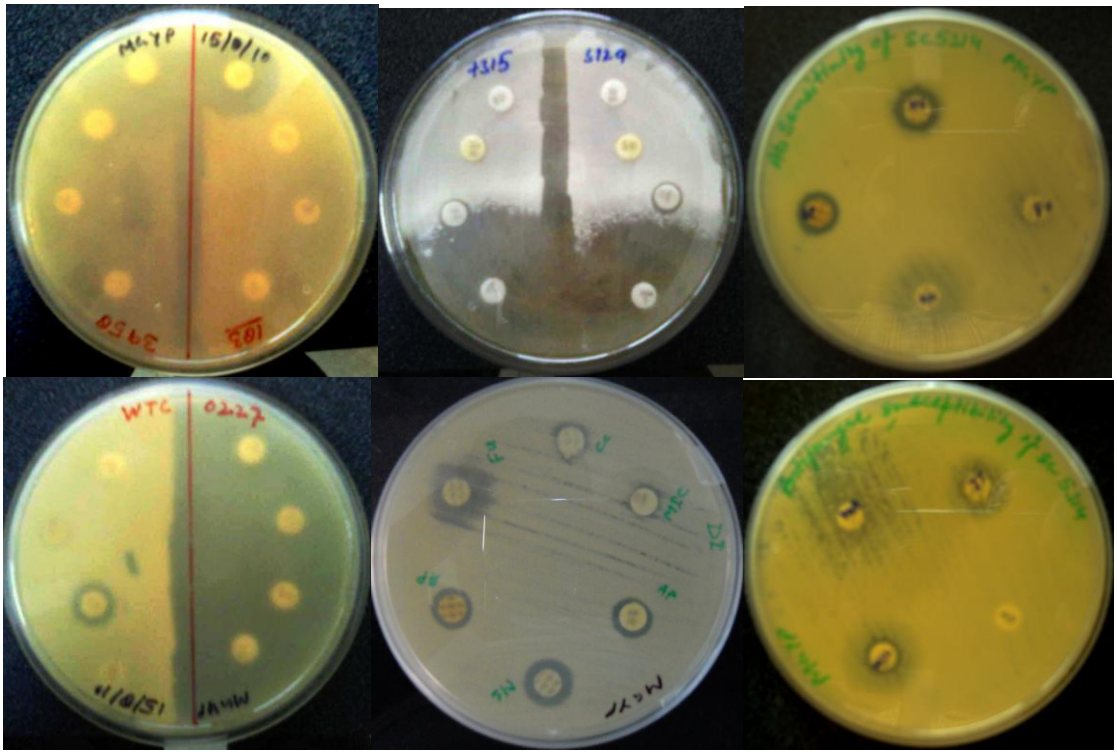


Figure 8. Antifungal antibiotics susceptibility plates of different *C. albicans* strains.

4.3. Antimicrobial Peptide/Protein Production by *E. faecalis*

4.3.1. Antimicrobial Potential of *E. faecalis*

The Antarctic penguin rookery isolate APR210, identified as *E. faecalis*, exhibited very satisfactory activity (>18-mm zone of inhibition) against several multidrug resistant *C. albicans* strains (NCIM 3471, MTCC 183, MTCC 7315, MTCC 227, MTCC 3958, SC 5314) and a wild-type diabetic isolate of *C. albicans* (DI, 13-mm zone of inhibition). Initially, APR210 showed mild activity against *P. aeruginosa* (15-mm zone of inhibition). It was also biologically active against a wild-type *Bacillus* species that grew as a contaminant on the assay plate and against 1 another isolated strain, AGM108-5. The biological activity of *E. faecalis* is summarised in Table14. The plates illustrating the biological activity of APR210 are shown in Figure 9.

Table 14. Antimicrobial spectrum of *E. faecalis* (ND, activity not detected).

Strains	**Identified organisms**	**Pathogens**	**Zone of inhibition**
APR210	*Enterococcus faecalis*	*Candida albicans*	
		NCIM3471	20 mm
		MTCC183	21 mm
		MTCC7315	21 mm
		MTCC227	18 mm
		MTCC3958	18 mm
	Dialysed Concentrate	MTCC183 & MTCC7315,	55 mm, 47 mm
		Wild type *C. albicans* (DI)	13 mm
		Wild type *C. albicans* (WI)	ND
		SC 5314 *C. albicans*	18mm
		Wild type Bacillus species	12 mm
		P.aeruginosa	15 mm
		AGM (108-5)	19 mm
		C. krusei (NCIM 3129)	ND
		C.neoformans (NCIM 3541)	ND
		C.glabrata	ND
		Aspergillus flavus (NCIM 857)	ND
		Aspergillus niger	ND
		Ps. Putida	ND
		E. faecalis (MTCC2080)	ND

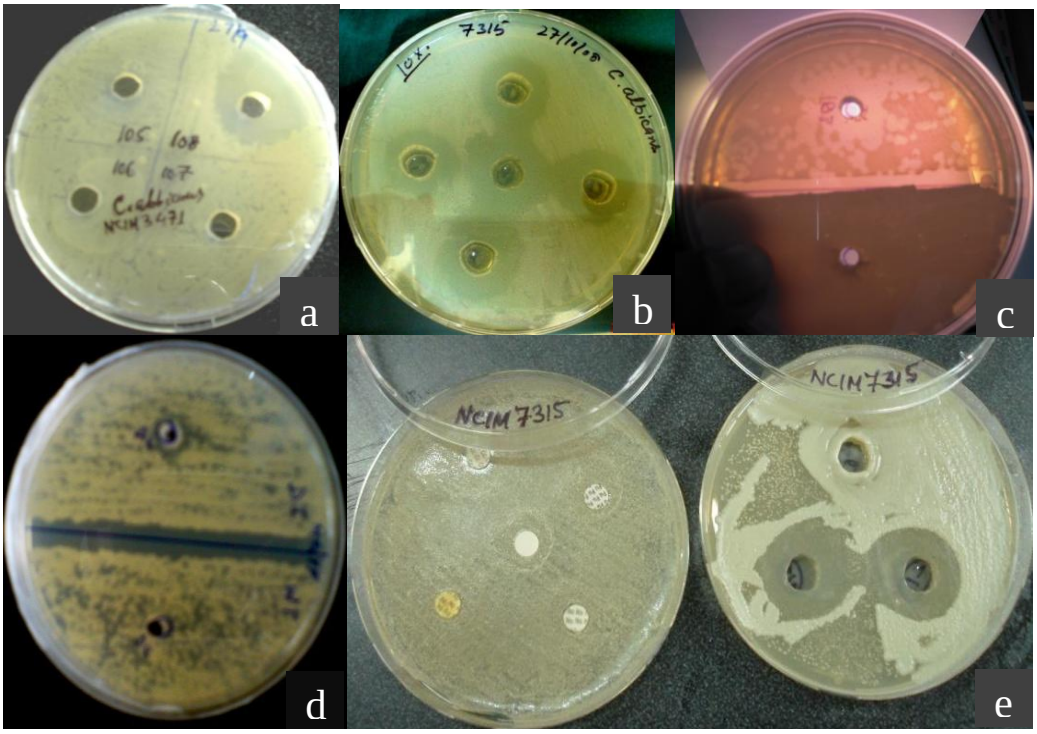


Figure 9. Biological activity plates of *E. faecalis* against several indicator organisms. (a) Biological activity of AGM108-5 against NCIM 3471. (b) Biological activity of *E. faecalis* CFS against MTCC 7315. (c) Extracellular antimicrobial compound production by *E. faecalis* (d) Biological activity of *E. faecalis* against the wild-type diabetic isolate (DI) (e) Antifungal drug resistance shown by MTCC 7315 and the biological activity of *E. faecalis* against MTCC 7315.

4.3.2. Optimization of Physical and Chemical Parameters for the Production of ACP

4.3.2.1. Optimization of temperature and incubation period

Optimal production of anti-*Candida* protein (ACP) from *E. faecalis* was observed at 48 h of incubation at 14–35°C and pH 7.4 ± 0.2 with control, and the antimicrobial activity began to dwindle after 48 h. The biological activity was recorded to be very low after 96 h of incubation, and the activity disappeared at 120 h of incubation (Figure 10). The *C. albicans* strains MTCC 3958 and 183 were used as indicator strains for the antimycotic assay.

When the test strain was incubated at different temperatures, optimal ACP production was recorded at 14–35°C, with an estimated activity of 1600 AU/ml against MTCC 3958. After 35°C, the antimycotic activity diminished (Figure 11). At 45°C and a constant pH of 7.4 ± 0.2, very mild activity was observed at 48 h of incubation, whereas the control strain incubated at 15°C showed maximal antimicrobial activity after 48 h.

4.3.2.2. Optimization of media pH

Optimization at varying pH values revealed that the optimum inhibitory activity and ACP production of *E. faecalis* was observed at pH 7.0 and 8.0, followed by pH 6.0, during the later growth stage after 48 h of incubation (Figure 12). ACP production and antimycotic activity started decreasing after 48 h, during the late growth stage.

The control strain showed optimal ACP production at pH 7.4 ± 0.2, followed by pH 8.0, at 48 h. ACP production was observed at pH values between 6.0 and 8.0, but there was no activity in the pH range 2.0–4.0.

4.3.2.3. Optimization of inoculum size

Optimization of inoculum size revealed that maximal ACP production (1600 AU/ ml) and the maximum activity of the test strain was observed when the inoculum size was 1.0% (Figure 13).

ACP production was commensurate with the size of the inoculum, as increments in the freshly grown inoculum up to 1% produced progressively larger zones of inhibition (Figure 13). However, a marked decrease in the production of antimicrobial substances resulted in reduced inhibition against 2 indicator strains, MTCC 183 and 3958.

4.3.2.4. Optimization of NaCl concentrations

At a constant pH of 7.4 ± 0.2 and a temperature of 14°C, 1–8% (w/v) of sodium chloride was added to mTS medium. Moderate increase in antimycotic activity was found with NaCl concentrations of 2–5%, and maximal activity was seen in 4% NaCl, in comparison to the control (Figure 14). However, further increases in NaCl concentration resulted in an inhibitory effect, as ACP activity decreased drastically at a concentration of 7%. Growth was not observed in 8% NaCl, nor was the inhibitory activity of the cell-free supernatant (CFS) obtained. It was evident that NaCl concentration of 4–5% enhanced the antimycotic effect (Figure 14).

4.3.2.5. Optimization of different media

The growth and antimycotic activity of *E. faecalis* was monitored using different media. Maximal activity was noted in mTSB (1600 AU/ ml) followed by TSB and MGYP, after a 48-h incubation. The least activity was found in MR-VP medium after incubation for 96 h (Figure 15).

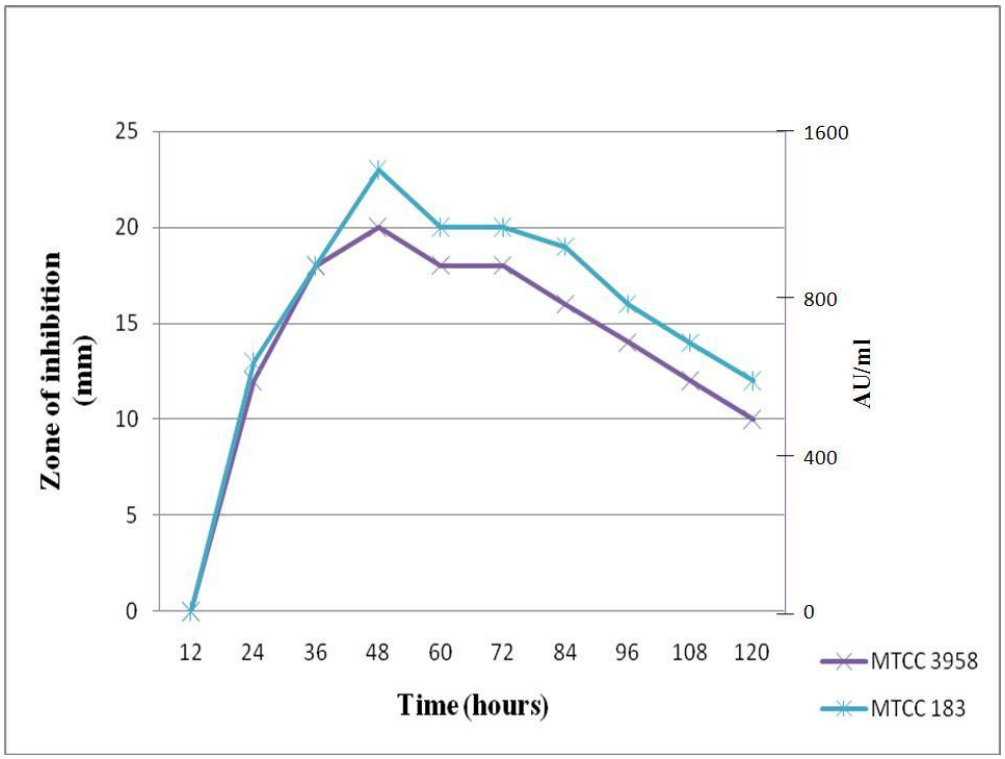


Figure 10. Effect of incubation times on the ACP production by *E. faecalis* at 14°C.

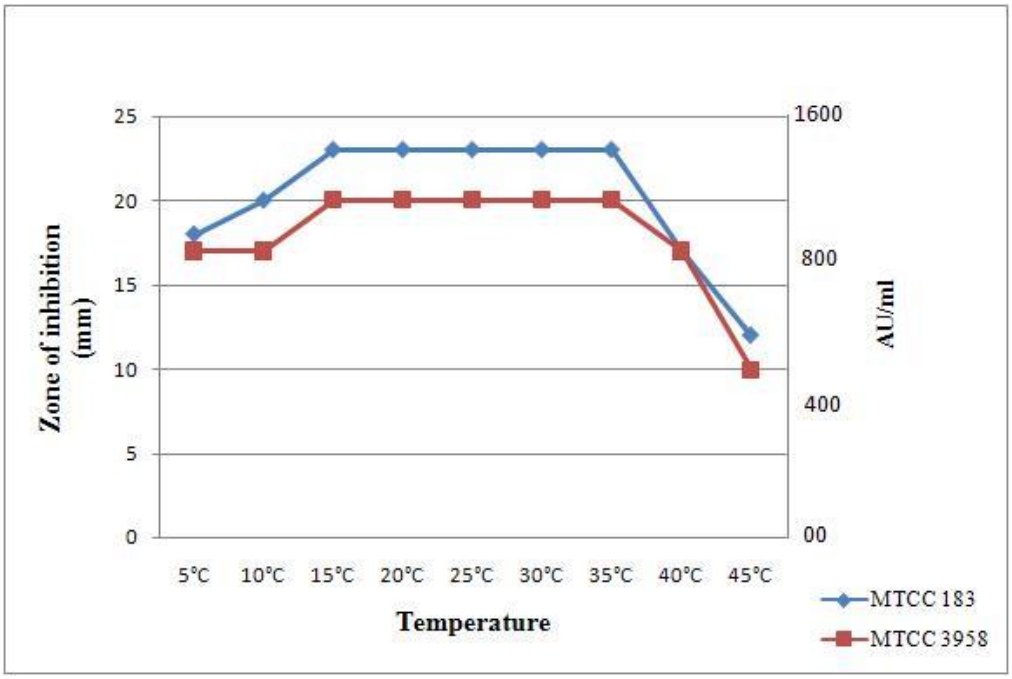


Figure 11. Effect of different incubation temperatures on the ACP produced by *E. faecalis*.

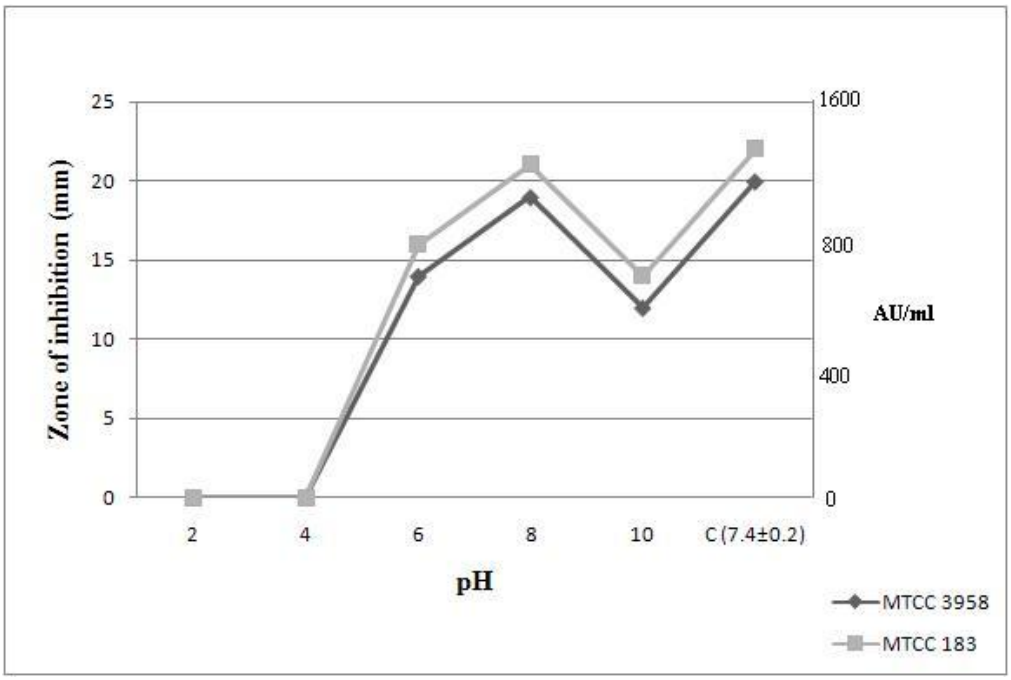


Figure 12. Effect of media pH on the ACP produced by *E. faecalis* incubated for 48 hr.

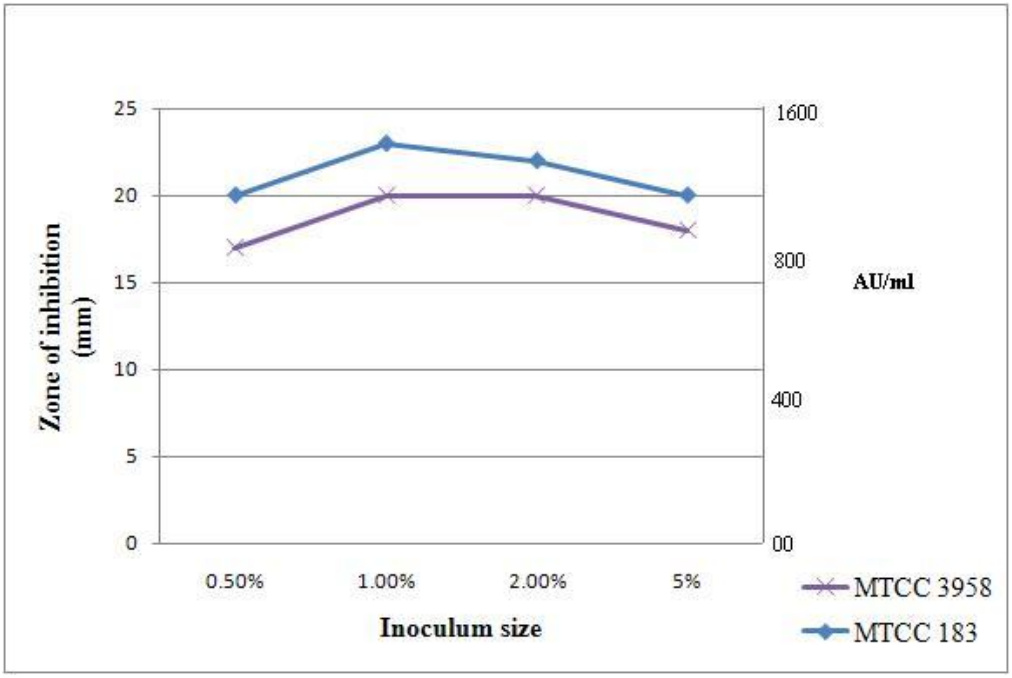


Figure 13. Effect of inoculum size (10^8 CFU/ ml) on the production of ACP by *E. faecalis* after 48 hr of incubation at 14°C.

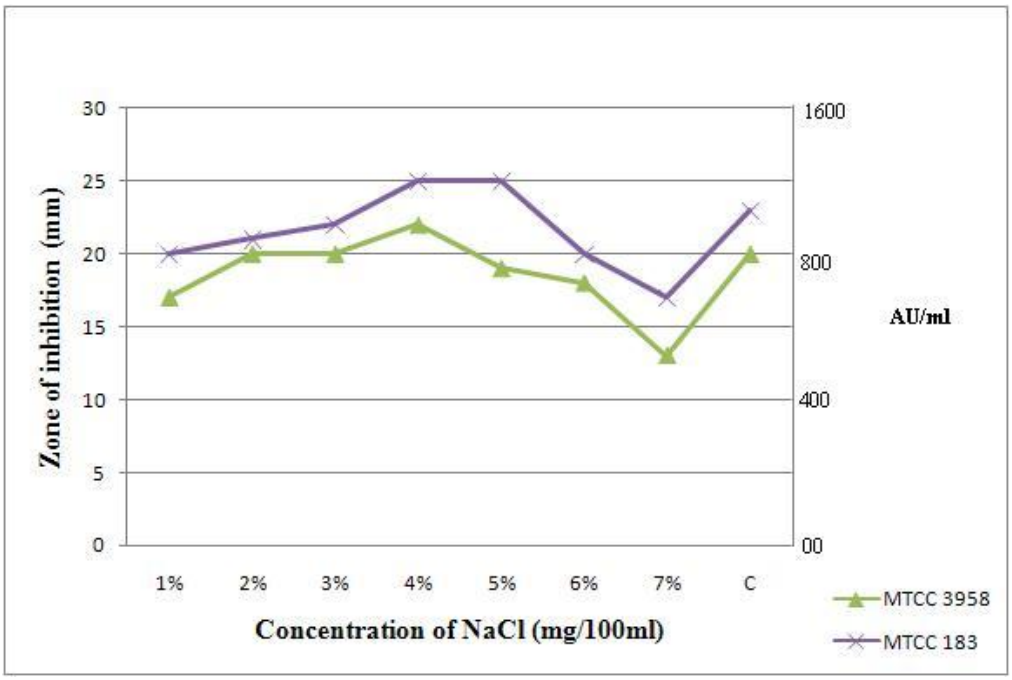


Figure 14. Effect of NaCl concentration on the ACP production by *E. faecalis* collected after 48 hr

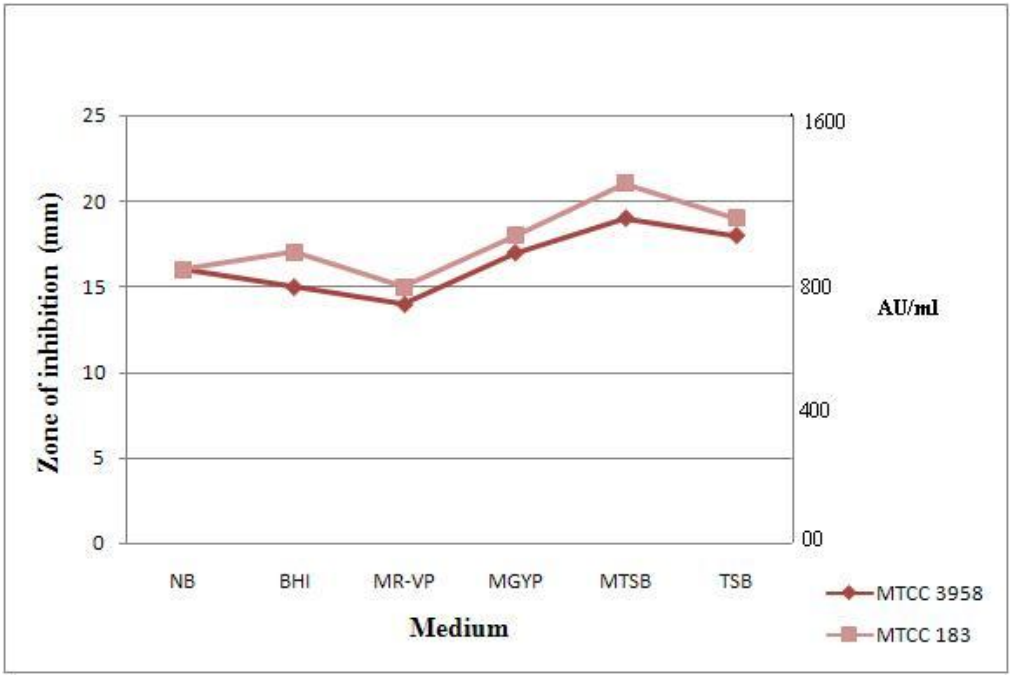


Figure 15. Production and activity of ACP in different growth media collected after 48 hr of incubation.

4.3.3. Kinetics of ACP Producing *E. faecalis*

Biomass and ACP production by *E. faecalis* in mTS broth was analyzed at an incubation temperature of 14°C (Figure 16). This strain reached the stationary phase after 20h and prolonged incubation promoted the degradation of the ACP but no lysis of biomass up to 56 hr. It was determined that no ACP was produced for up to 8h at 14°C but it was produced during active growth phase, and its concentration reached a maximum at 48 h, in the middle of the stationary phase. The highest activity (1600 AU/ml) against *C. albicans* MTCC 183 was recorded at 44–48 h of incubation and decreased thereafter. Mild biological activity was recorded at up to 96 hr of incubation, and further incubation up to 120h resulted in complete loss of activity. The pH plummeted during the exponential phase, probably as a consequence of the strong production of acid associated with growth.

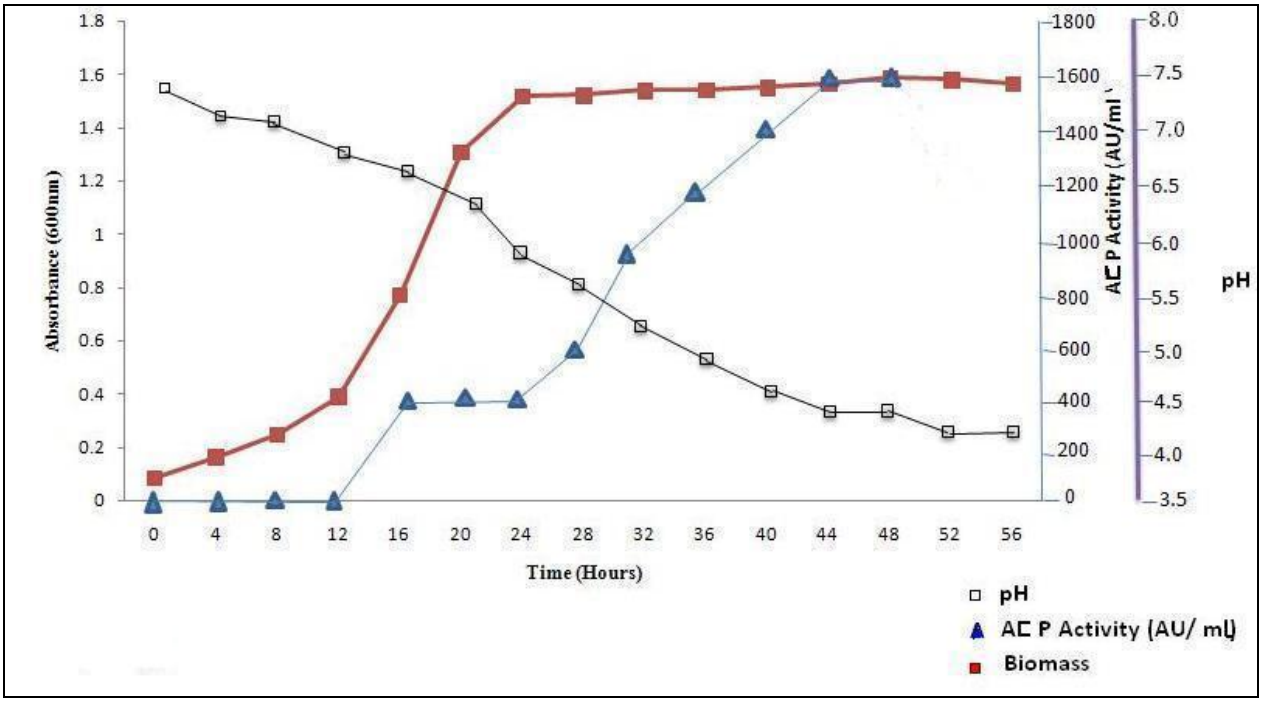


Figure 16. Production of biomass and antimicrobial substance by APR 210 and changes in pH during incubation. ▫ indicates changes in pH; ▲ indicates variation in ACP activity; ■ indicates absorbance at 600 nm.

4.4. Characterization of Anti-*Candida* Protein Produced by *E. faecalis*

4.4.1. Effect of Hydrolytic Enzymes on the Biological Activity of ACP

The ACP was found to be fully sensitive to proteinase K and partially to pronase E (Table 15, Figure 17), confirming its proteinaceous nature, however, its resistance to pepsin, lysozyme, and trypsin indicated that it might be composed of cyclic peptides that contain unusual amino acids and are therefore more resistant to protease hydrolysis (Bizani *et al*. 2002). These results suggested that this antimycotic peptide can possibly survive in the intestinal environment and may therefore be administered in food (Jianhua *et al*. 2009). On the other hand, the absence of any effect of α-amylase and lipase on antimycotic activity suggested that the AMP/ACP might not be glycosylated and might not contain a lipid moiety.

Table 15. Proteolytic digestion of the dialyzed concentrate of ACP in presence of several hydrolytic enzymes. (+, no loss; –, partial loss; – –, complete loss of biological activity in the presence of enzyme; *, 2.0 mg/ml).

Enzymes (1.0 mg/ml)	**Buffers**	**Activity**
Trypsin	50 mM Tris pH 8.0	+
Pronase E	10 mM Sod.Phosphate pH 7.0	-
Proteinase K	50 mM Tris HCl pH 7.5	--
Pepsin	20 mM Tris HCl pH 2-4	+
α-Amylase	50 mM Sod.Phosphate pH 7.0	+
Lipase	20 mM Sod.Phosphate pH 7.0	+
Lysozyme*	50 mM Sod.Phosphate pH 7.0	+

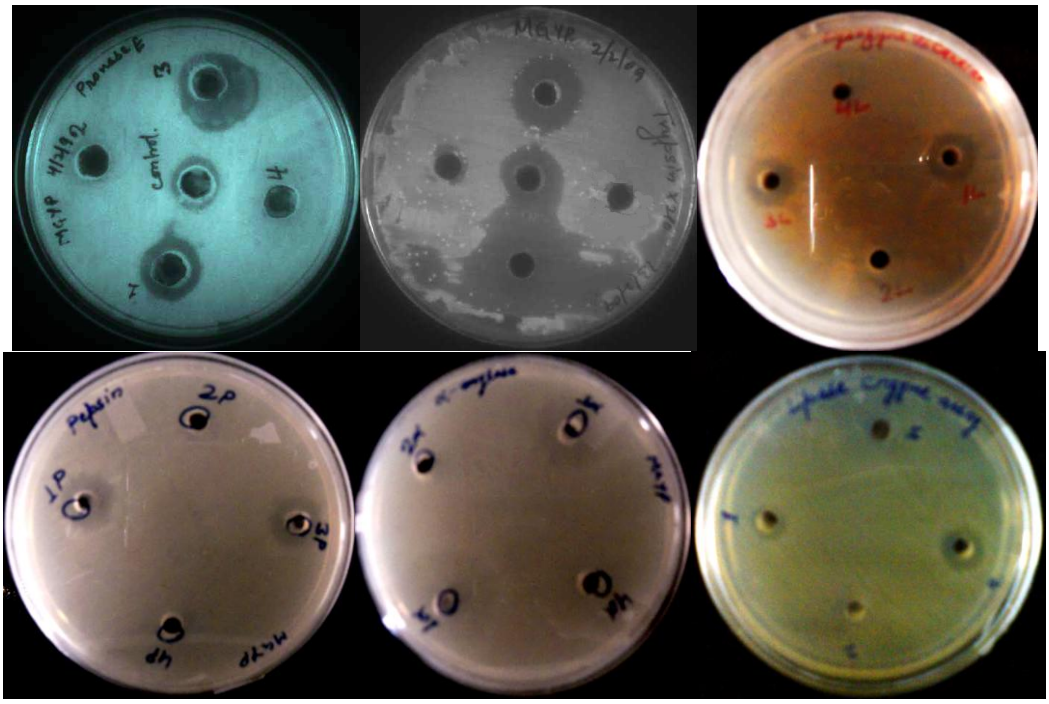


Figure 17.Sensitivity of ACP to different hydrolytic enzymes. Well no. (1) Enzymes with appropriate buffers, incubated in *E. faecalis* CFS. (2) Enzymes in appropriate buffers. (3) *E. faecalis* CFS alone. (4) Enzyme buffers with mTS broth.

4.4.2. Effect of Different pH on the Activity of ACP

The antimycotic property of ACP also remained unaffected in the pH range 6.0–8.0. However, the activity was reduced by 50% at pH 5.0 and 9.0, whereas complete loss of activity was seen at pH 2.0, 4.0, and 10.0 (Table 16). These results are similar to that reported for the bacteriocin produced by *E. mundtii* (Ferreira *et al*. 2007). Several bacteriocins produced by enterococci are known to exhibit stability in a wide pH range (Losteinkit *et al*. 2001).

Table 16. The effect of pH on the activity of ACP. –, complete loss of activity; +, partial activity (14-mm zone of inhibition); ++, complete activity (>18 mm zone of inhibition). (Shekh *et al*. 2011).

pH	Activity
2.0	–
4.0	–
5.0	+
6.0	++
7.0	++
8.0	++
9.0	+
10.0	–

4.4.3. Thermal Stability of ACP

The activity of CFS was stable upon treatment at different temperatures up to 90°C for 20 min, but the activity was lost after boiling and autoclaving (Table 17).

Table 17. Heat tolerance of the antimycotic protein produced by *E. faecalis* (+, retention of biological activity; –, loss in biological activity).

Temperature and time	Activity
37ºC for 90 min	+
60ºC for 30 min	+
60ºC for 90 min	+
90ºC for 20 min	+
100ºC for 30 min	–
100ºC for 90 min	–
121ºC for 15 min	–

4.4.4. Effect of Different Organic Solvents, Surfactants and Storage

The antimycotic peptide, ACP, remained fully active when treated with different surfactants and organic solvents, as mentioned in "Materials and Methods". The activity was enhanced by 33.4% in presence of SDS (1.0% w/v), long-term storage (1 year) at temperatures of -80°C did not affect the antimicrobial activity (98%) but a slight reduction (20%) in activity was seen at 4°C and -20°C (Table 18). The bioassay result plates are given in Figure 18.

Table 18. Effect of organic solvents, surfactants and storage on the biological activity of ACP. (+, No loss in biological activity. ++, increase in biological activity).

Treatment		Biological Activity
Organic Solvents		
Methanol	(25%)	+
Ethanol	(25%)	+
Iso-propanol	(10%)	+
Hexane	(25%)	+
Formaldehyde	(10%)	+
Chloroform	(10%)	+
Acetone	(10%)	+
Acetonitrile	(70%)	+
Detergents & Other Reagents		
Triton X-100	(1% v/v)	+
Tween-20	(1% v/v)	+
SDS	(1% w/v)	++
Urea	(1% w/v)	+
EDTA	(1% w/v)	+
PMSF	(1% v/v)	+
β-Mercaptoethanol	(1m M)	+
DTT	(0.1 M)	+

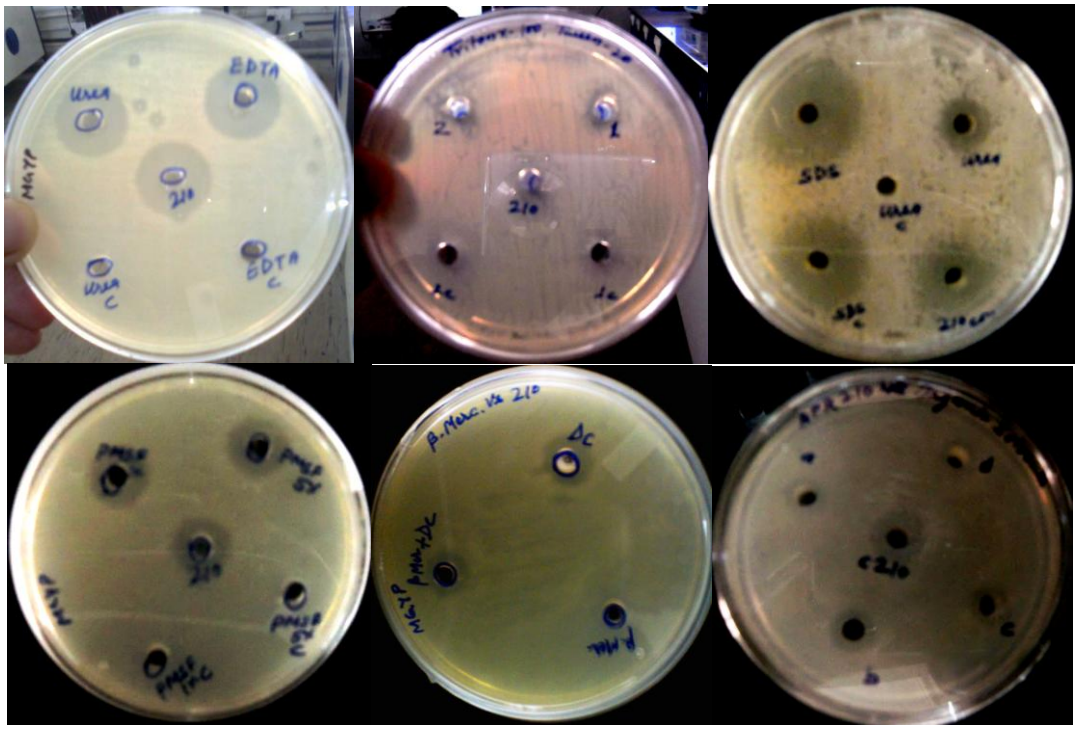


Figure 18. Effect of surfactants and organic solvents on the activity of ACP. Dialyzed concentrate without surfactants or organic solvents was used as the control.

4.5. Partial Purification of ACP

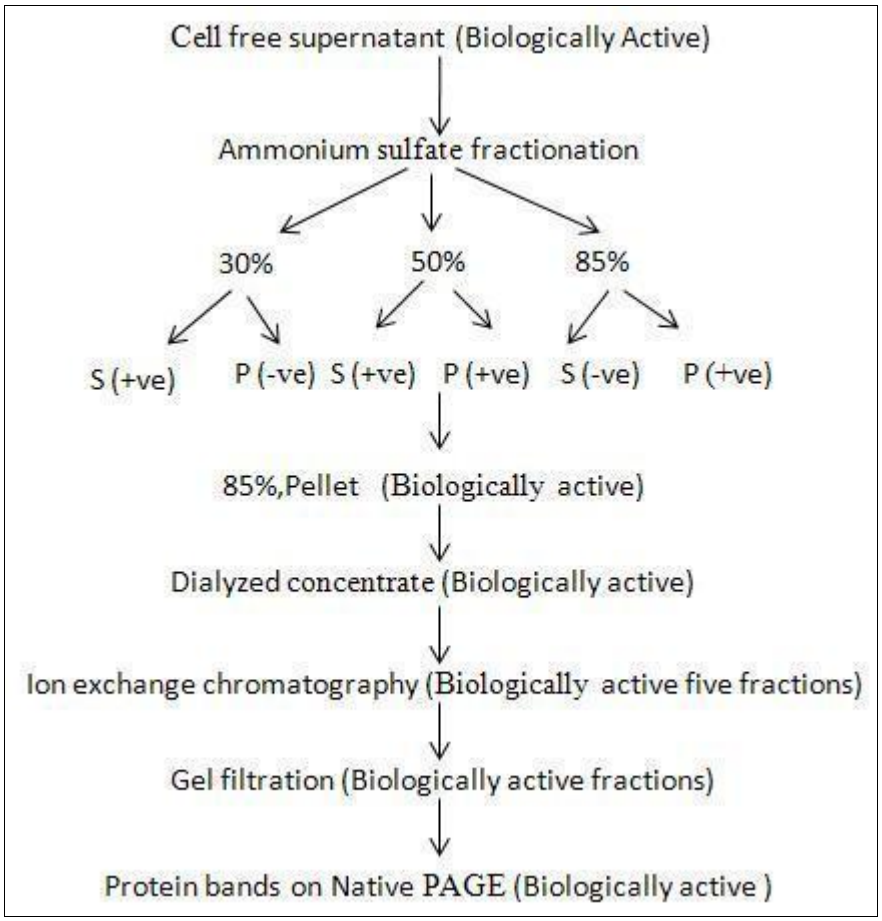


Figure 19. Summary of the purification of ACP. S (+ve) represents biological activity, and S (–ve) indicates no activity in supernatant. P (+ve) represents biological activity and P (–ve) indicates no activity in pellet.

A simple protocol, including ammonium sulphate precipitation, dialysis, ion exchange chromatography, gel filtration, and ultrafiltration, was followed for the purification of antimycotic protein. A summary of the purification procedure is given in Figure 19. The highest antifungal activity against different strains of *C. albicans* was present mainly in the fraction precipitated with 85% ammonium sulphate (Figure 20). Fractions precipitated with 30 and 50% ammonium sulphate exhibited weak inhibition. Supernatant obtained after 85% ammonium sulphate precipitation clearly did not exhibit any antifungal activity (Figure 20).

It is clear from Table 19, that at the first step, ammonium sulphate precipitation resulted in an around 2-fold increase in specific activity. On a DEAE Sepharose column, the bound ACP was eluted with increasing concentrations of NaCl in sodium phosphate buffer, pH 8.0. As seen in Figure 22, 5 biologically active fractions were obtained with 0.28–0.34 M NaCl. The 5 fractions (31–35) showed biological activity against *C. albicans* MTCC 183 (Figure 21a) and the pooled fractions showed mild antimycotic activity against SC 5314 (Figure 21b).

The chromatogram of antimycotic protein eluted from DEAE Sepharose and the absorbance at 280 nm of the fractions is shown in Figure 22; at this stage, the maximum activity of the pooled fractions was 1600 AU/ml. After anion exchange chromatography, around 17-fold increase in specific activity was recorded, whereas after gel filtration with Sephadex G-75, the recovery was around 22-fold. The chromatogram of fractions collected during gel filtration on Sephadex G-75 is presented in Figure 23. Based on the purification steps summarised in Table 19, it was concluded that the total recovery percentage of active antimycotic protein was only 0.45%.

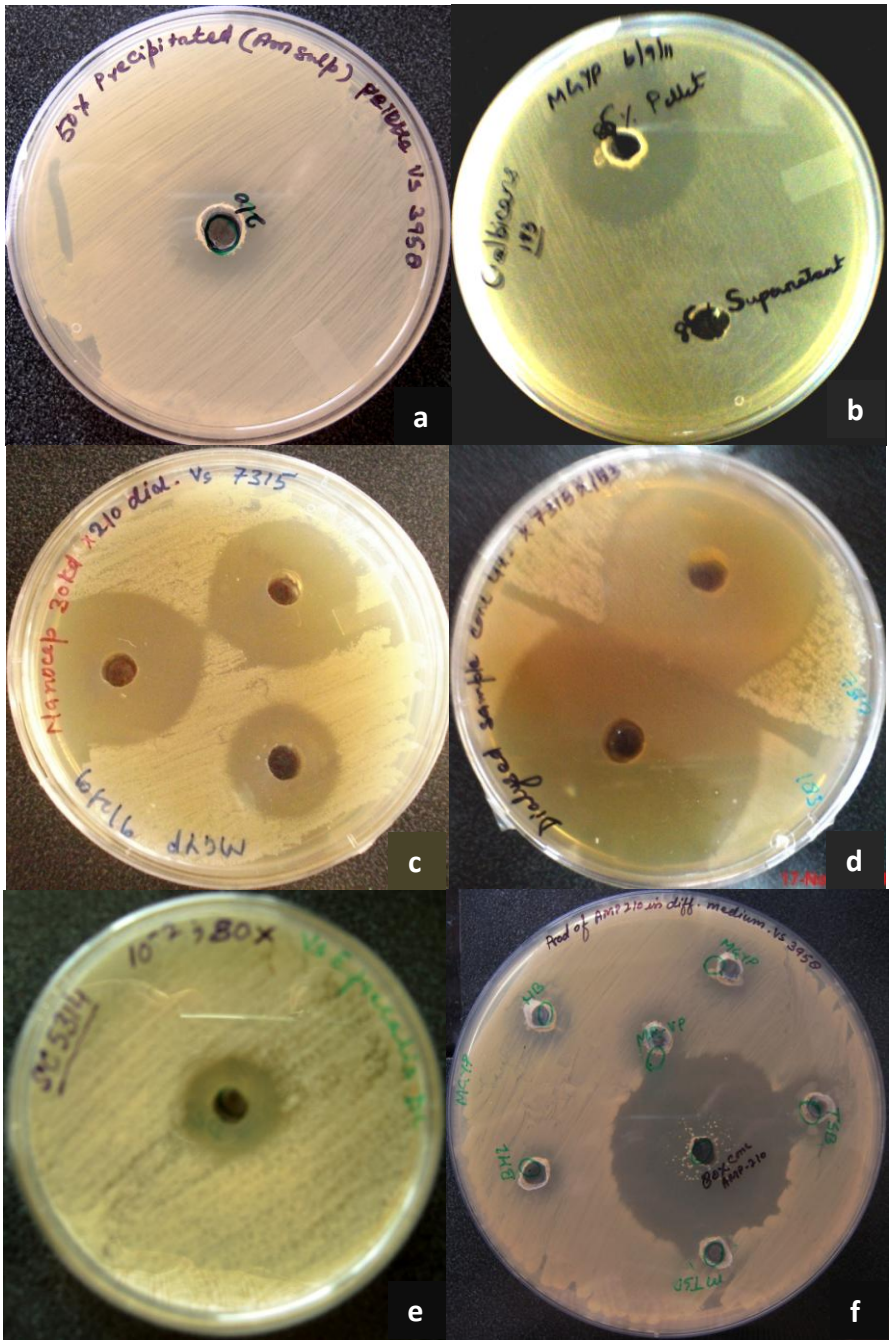


Figure 20. Antimycotic activity in pellets obtained by precipitation with (a) 50%, (b) 85% ammonium sulphate in 20 mM sodium phosphate buffer. No activity was detected in the supernatant. (c) Dialysed concentrate (DC) against MTCC 7315 ultafiltered by 30kDa Nanocep unit. (d) Activity of DC (80X) against MTCC 183 and MTCC 3958. (e) Activity of DC against SC 5314 and (f) activity of DC (80X) against MTCC 3958.

Table 19. Summary of the purification procedure; specific activity, purification factor, and percent recovery at each step.

Purification stage	Volume(ml)	Activity (AU/ml)	Protein (mg/ml)	Specific activity (AU/mg protein)	Purification Factor	Recovery (%)
Culture Supernatant	400	1600	0.4025	39751	1	100
Ammonium sulfate fr.& dialysis	10	3200	0.0444	72072	1.8	11
Ion Exchange Chromatography	6	1600	0.0023	695652	17.5	0.57
Gel Filtration	2	1600	0.0018	888888	22.4	0.45

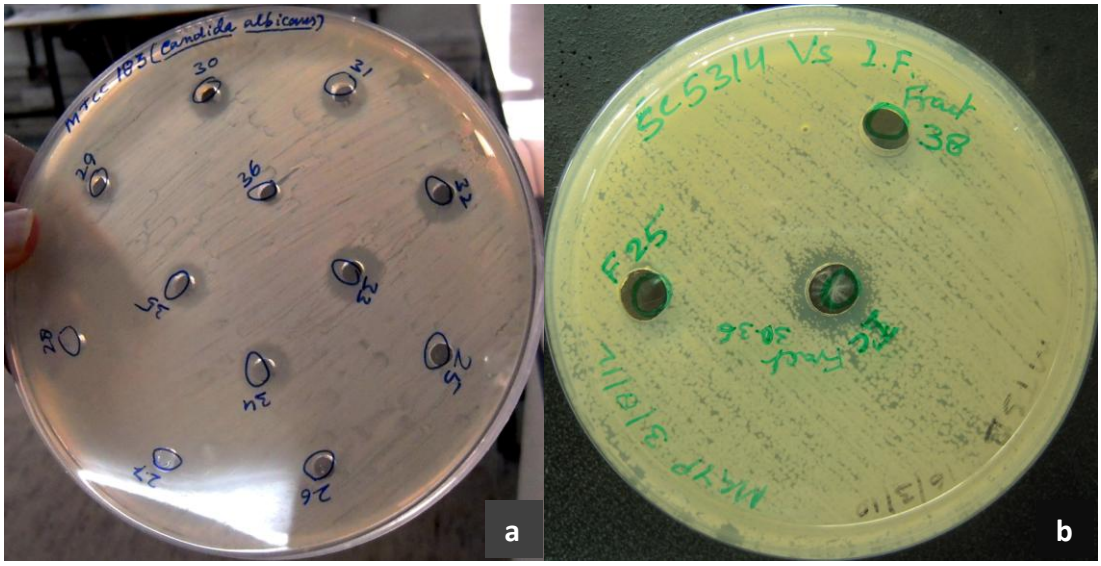


Figure 21(a) Antifungal activity of the fractions against *C. albicans* (b) Antifungal activity of pooled fractions against SC 5314, collected during anion exchange chromatography using DEAE Sepharose matrix.

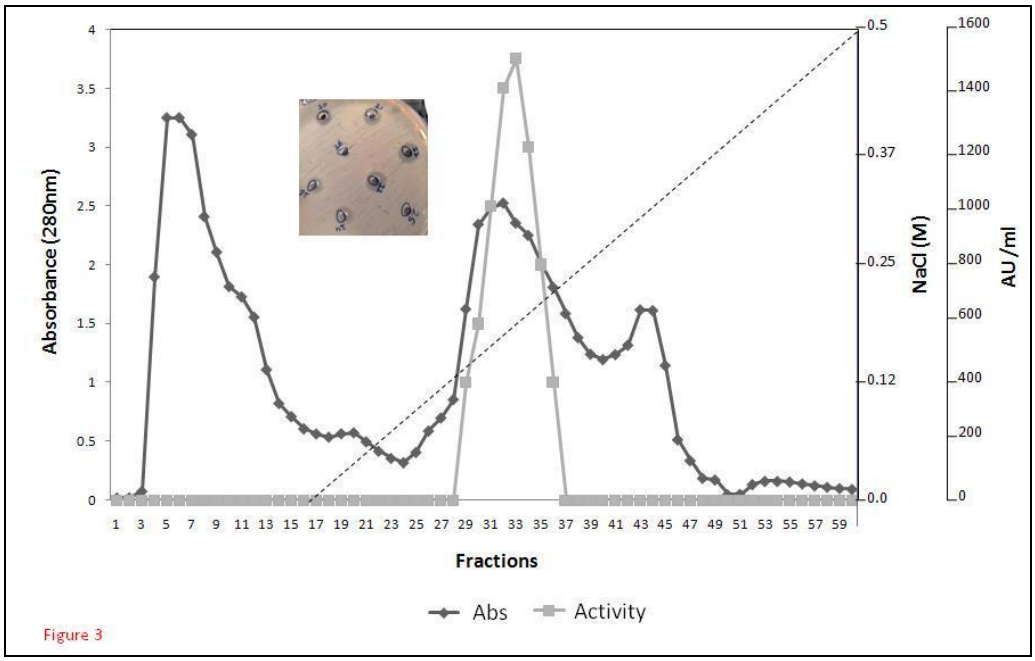


Figure 22. Chromatogram of the ACP produced by *E. faecalis* using DEAE Sepharose, absorbance of fractions at 280 nm and activity (AU/ml).

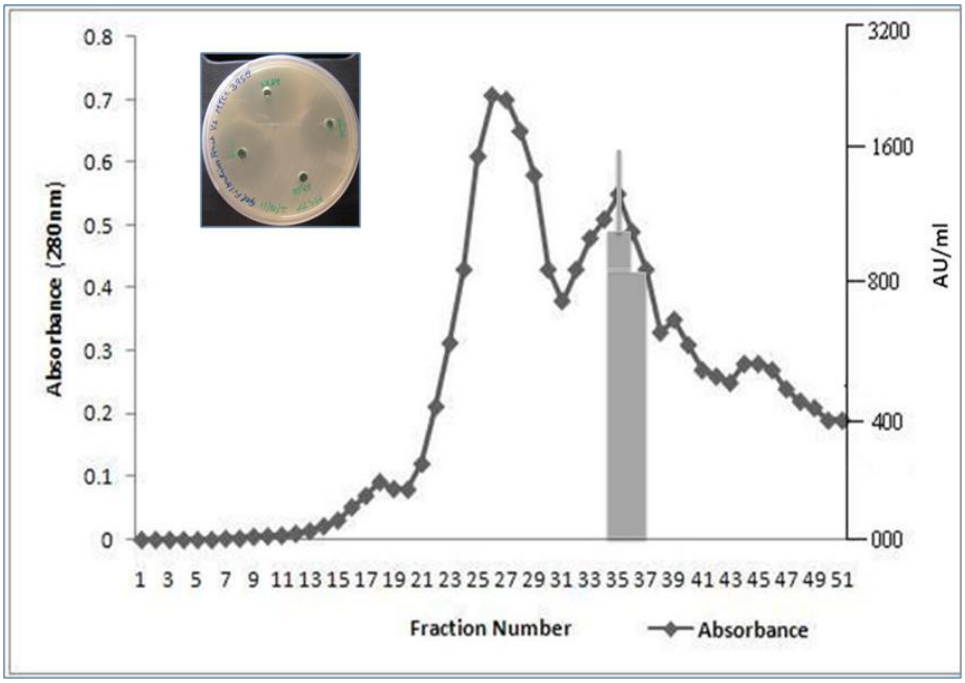


Figure 23. Chromatogram of fractions collected during gel filtration on a Sephadex G 75 column. The line represents the absorbance at 280 nm. Biological activity against MTCC 3958 was found in fractions 31–34 (inset picture). ■ represents AU/ml.

4.5.1. Molecular Weight of ACP on Tricine Native and SDS-PAGE.

The molecular weight of antimycotic protein was determined based on standard molecular weight markers ranging from 6.5 to 97.4 kDa (GeNei, Bangalore, India). Protein bands resolved on 10% Tricine Native as well as SDS-PAGE gel at each step are shown in Figure 24 (a and b). ACP was around ~43 kDa, as also concluded using a 30 kDa ultrafiltration unit (Nanosep).

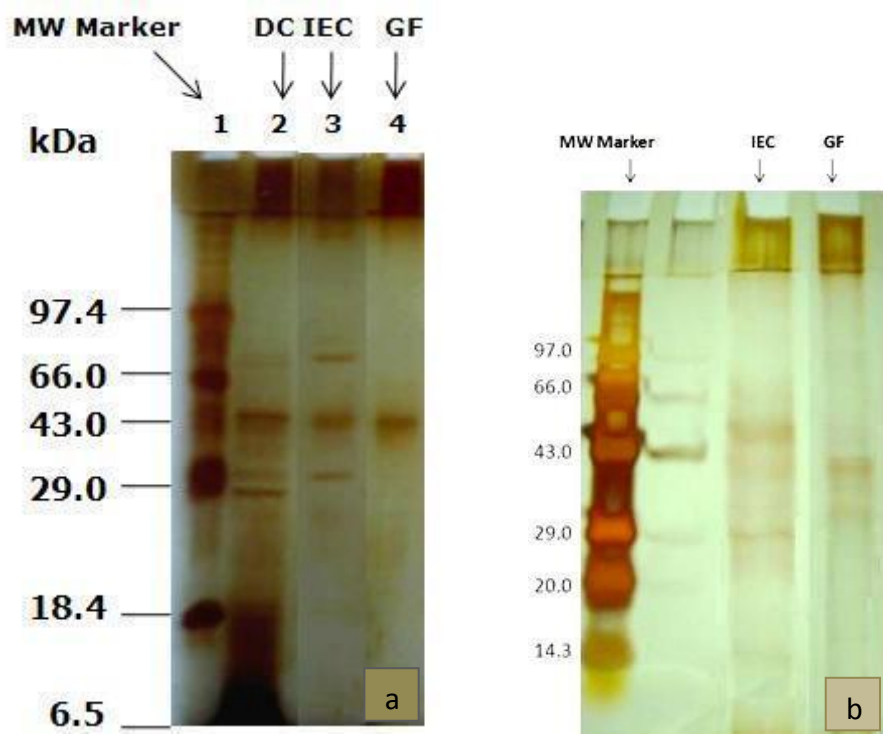


Figure 24. (a) Tricine Native -PAGE of various ACP preparations obtained by different protocols (silver-stained). Lane 1, molecular weight marker; Lane 2, dialyzed concentrate of CFS after 85% ammonium sulphate fractionation dissolved in 20 mM sodium phosphate buffer; Lane 3, pooled biologically active fractions obtained after anion exchange chromatography using a sodium chloride linear gradient (Fig 22); Lane 4, pooled fractions obtained after gel filtration with Sephadex G75. (b) Tricine SDS-PAGE profile of ion exchange and gel filtration fractions.

4.5.2. Direct Detection of Antimycotic Activity on Tricine Native PAGE

After gel filtration, partially purified biologically active pooled fractions (30 µl), were loaded onto a tricine gel containing 10% resolving and 5.0% stacking gels. A clear zone of inhibition on the *C. albicans* MTCC 3958 overlaid gel was visible in a Petri dish (Figure 25), and the silver-stained gel showed a corresponding band that was responsible for biological activity. Based on the protein molecular weight marker, the molecular mass of active protein was estimated to be around 43 kDa (Fig 25a). No biological activity of the bands was observed using Tris-glycine native PAGE (Shekh *et al*. 2012).

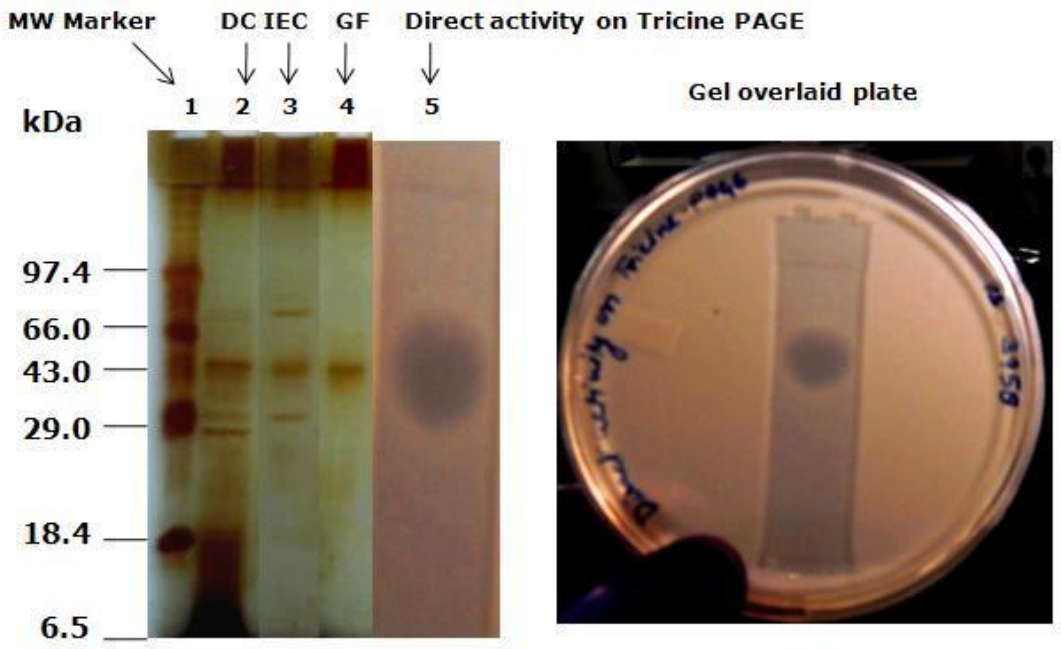


Figure 25. Inhibition zone by anti-*Candida* protein (ACP) on an overlay gel against 0.75% *C. albicans* MTCC 183. (a) Silver-stained gel showing each step of the purification process. (b) Semisolid gel-overlaid plate showing the zone of inhibition.

4.5.3. *De novo* Sequencing of ACP

The sequencing results for ACP are shown in Table 20 (refer Figures 26a, 26b, and 26c for *de novo* sequencing spectra). In the Table, the first column on the left contains the *m/z* of the parent ions, and the second column contains the deduced sequences. A maximum of 5 sequences (*i.e.* interpretations) are presented for any given ion. These are ranked based on their likelihood (0 to 4), with the ranking shown in the fourth column. The third column (titled Score) is also related to this ranking and shows the likelihood of any sequence amongst all possible interpretations for an ion. The last column denotes the quality of the raw spectrum for the ion in question. The closer this value is to 1, the higher the quality.

From the *de novo* sequence, the combined peptide with 40 amino acid residues was assembled. Individual peptides with *m/z* values of 718, 1039, and 601 were found (Table 20). The combined peptide did not contain any charged acidic residues (Asp, Glu). Hydrophobic amino acids constituted 42.5% (excluding Gly). The peptides did not significantly match any known proteins present in the MASCOT and BLASTp databases. The amino acid sequence of ACP (40 residues), obtained from the peptide fragments after digestion of the antimycotic protein with trypsin, was analyzed by MS/MS spectra using PEAKS Studio Version 4.5 SP2 (Bioinformatics Solutions) with subsequent *de novo* sequencing. The peaks obtained are indicated in the sequence below, and overlapping residues are shown in bold (Figures 26d).

Unfiltered BLAST searches using the *de novo* sequences did not identify any sequence with homology in the Protein Data Bank (PDB). Only a small patch of the sequence matched; for example, a WL motif that occurs 2 times in the enterocin 1071B amino acid sequence (Maldonado-Barragan *et al*. 2009) was found 4 times in WLPPAGLLGRCGRWFRPWL LWLQSGAQYKWLGNLFGLPGK in the combined *de novo* sequence (Figure 26d) of ACP. An earlier study on Ponericin W1 and W2 revealed the presence of the WL and GL motifs and the presence of hydrophobic residues (Shekh *et al*. 2012).

Table 20: *De novo* sequencing results of ACP

m/z	Peptide	Score (%)	Rank
718.29	WLPPAGLLGRCGR	95.0	0
718.29	WLPPAGLFCQCK	2.9	1
10,39.72	WFRPWLLWLQSGAQYK	77.0	0
10,39.72	FWRPWLLWLQSGAQYK	7.3	1
601.24	WLGNLFGLPGK	65.2	0
601.24	WLGNLFGLGPK	32.2	1

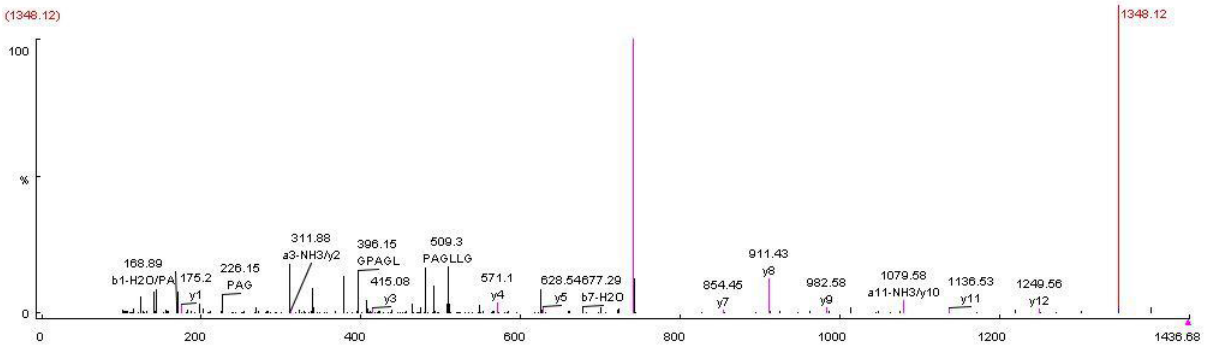


Figure 26 a. *De novo* spectra for the peptide at *m/z* 718.29: **WLPPAGLLGRCGR**

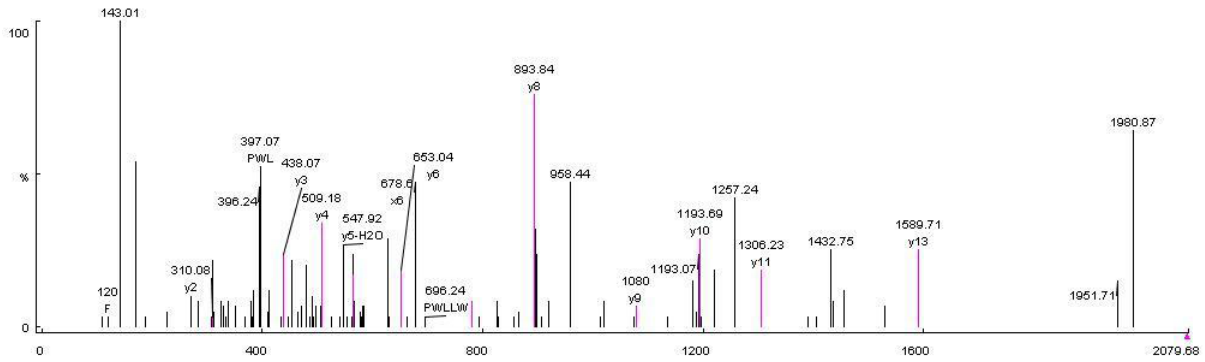


Figure 26 b. *De novo* spectra for peptide at m/z 1,039.72: **WFRPWLLWLQSGAQYK**

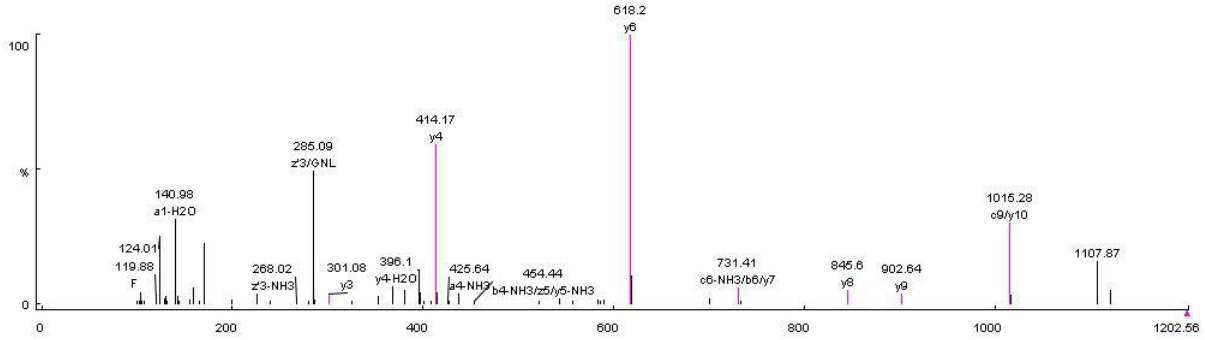


Figure 26 c. *De novo* spectra for the peptide at *m/z* 601.24: **WLGNLFGLPGK**

Figure 26 d. Combined *de novo* sequence of ACP consisting of the 3 peptides with *m/z* ratios of 718, 1039, and 601

Based on the peaks and matching with MASCOT search, the respective protein/peptide has no significant match with any protein/peptide present in the data base. It is highly considered that it might be new or novel antimycotic peptide produced by *E. faecalis*.

4.5.4. N-Terminal Amino Acid Sequencing of Partially Purified ACP

Table 21. N-terminal amino acid sequencing results of ACP obtained after 12 cycles.

Cycle Number	Amino Acid
1	D, G
2	E, P
3	V, G
4	Y, G
5	T, P
6	V, G
7	K
8	S + S'
9	G
10	D
11	S + S'
12	L

The first 12 N-terminal amino acid residues were identified by Edman degradation (Table 21). The first 6 amino acid peaks are given in Figure 27 (a, b, c, d, e and f). The minor sequence obtained from the twice-repeated N-terminal sequencing was GPGGPGKSGDSL, and the same partial sequence was matched for homology. Complete homology was not found using the NCBI BLAST tool. Analysis of the major N-terminal sequence DEVYTVKS(S+S') GDSL revealed the presence of S', suggesting that serine is modified; this is a feature of class I antibiotics (Begley *et al.* 2009). This sequence was found to be very similar to that of autolysin and a hypothetical protein of *E. faecalis* in BLASTp search.

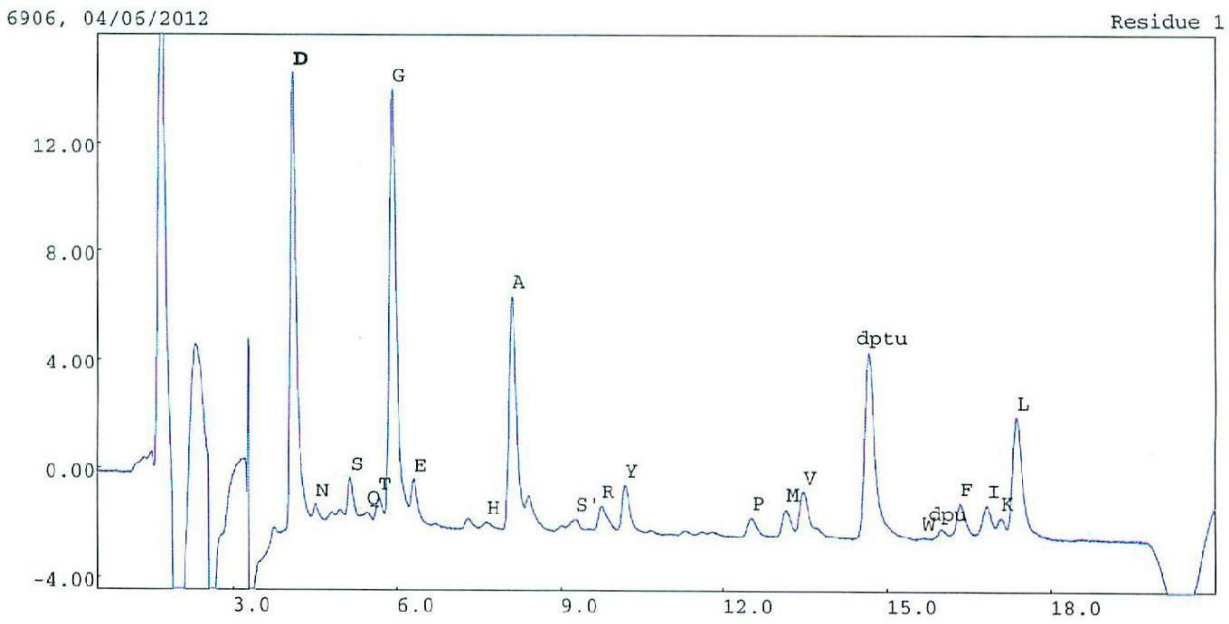


Figure 27 a. Residue 1

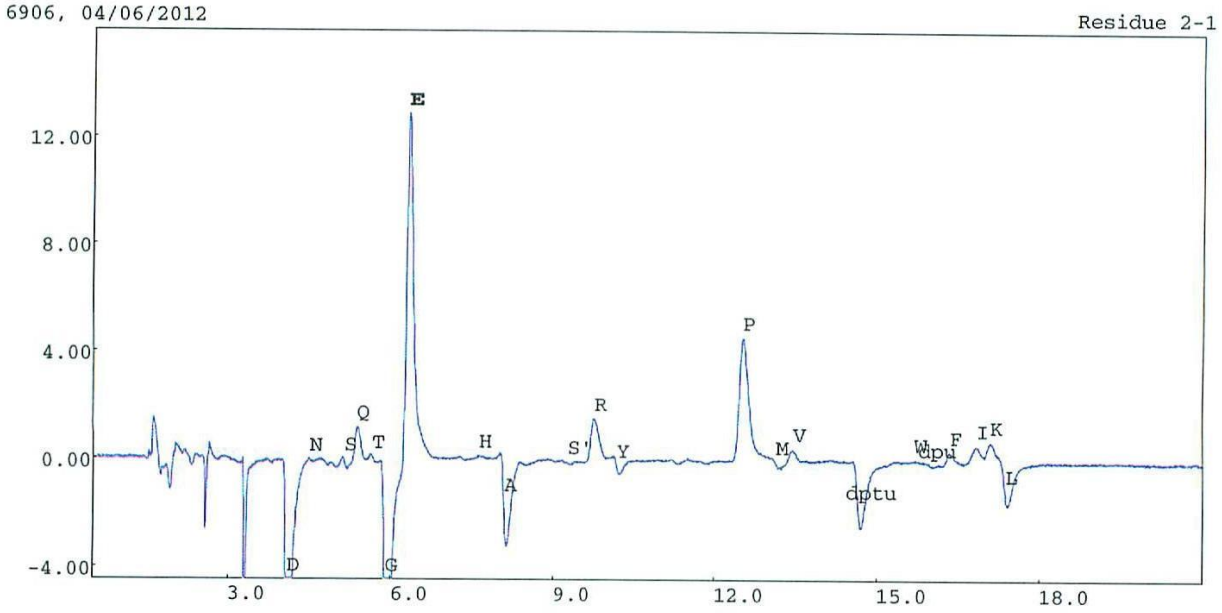


Figure 27 b. Residue 2

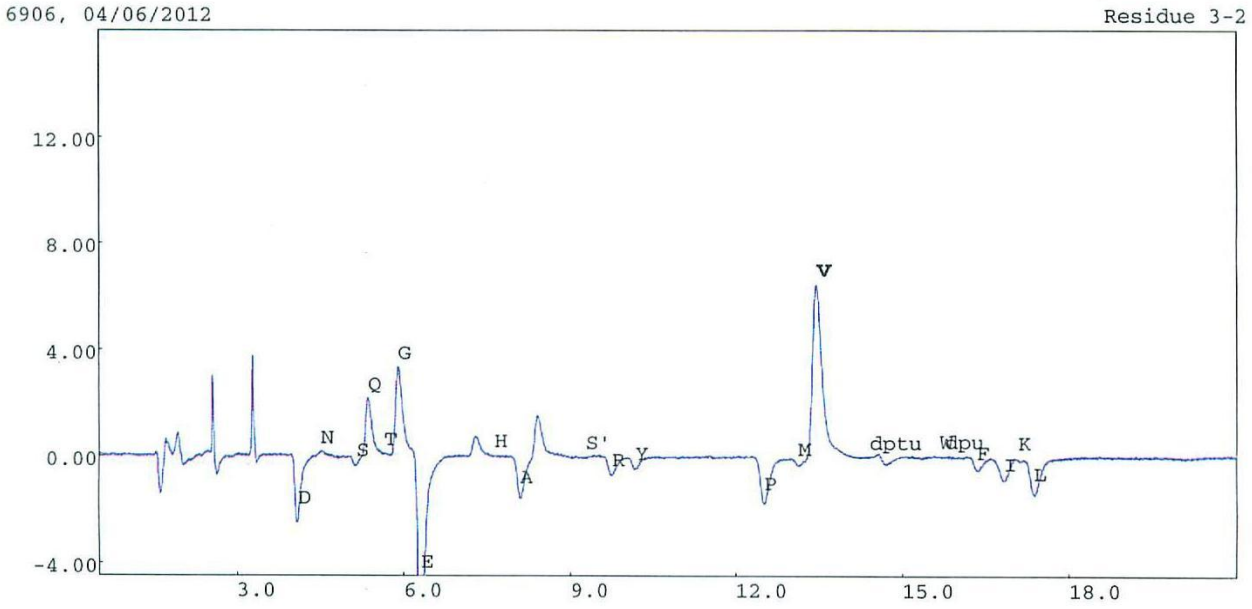


Figure 27 c. Residue 3

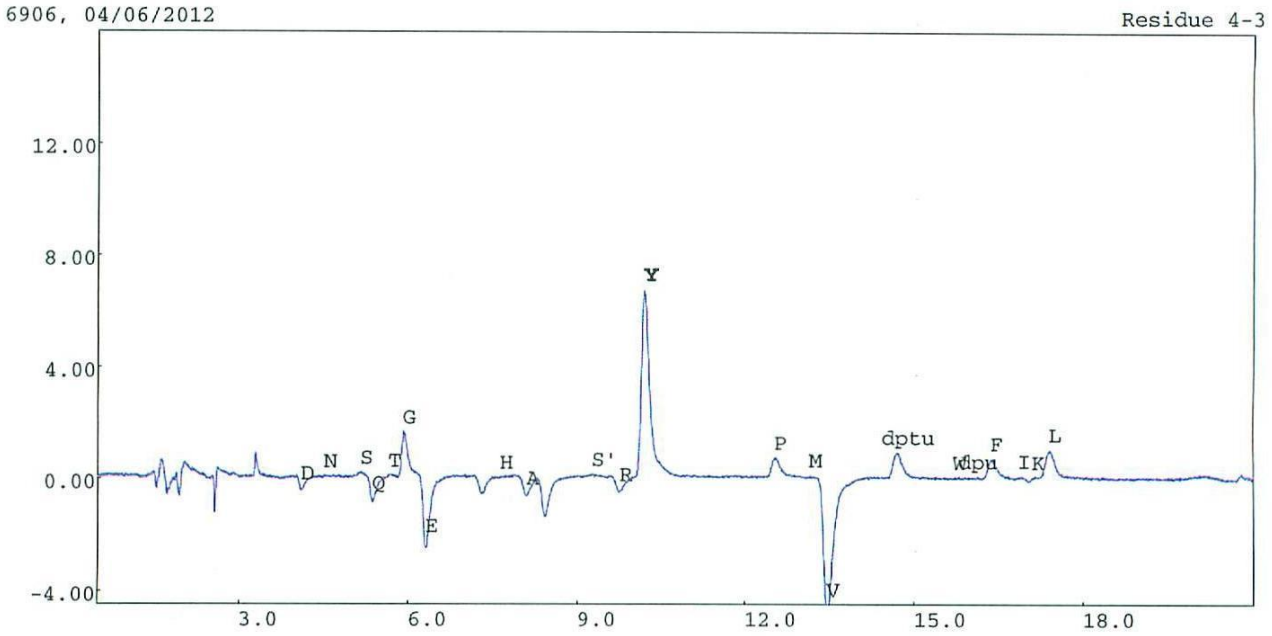


Figure 27 d. Residue 4

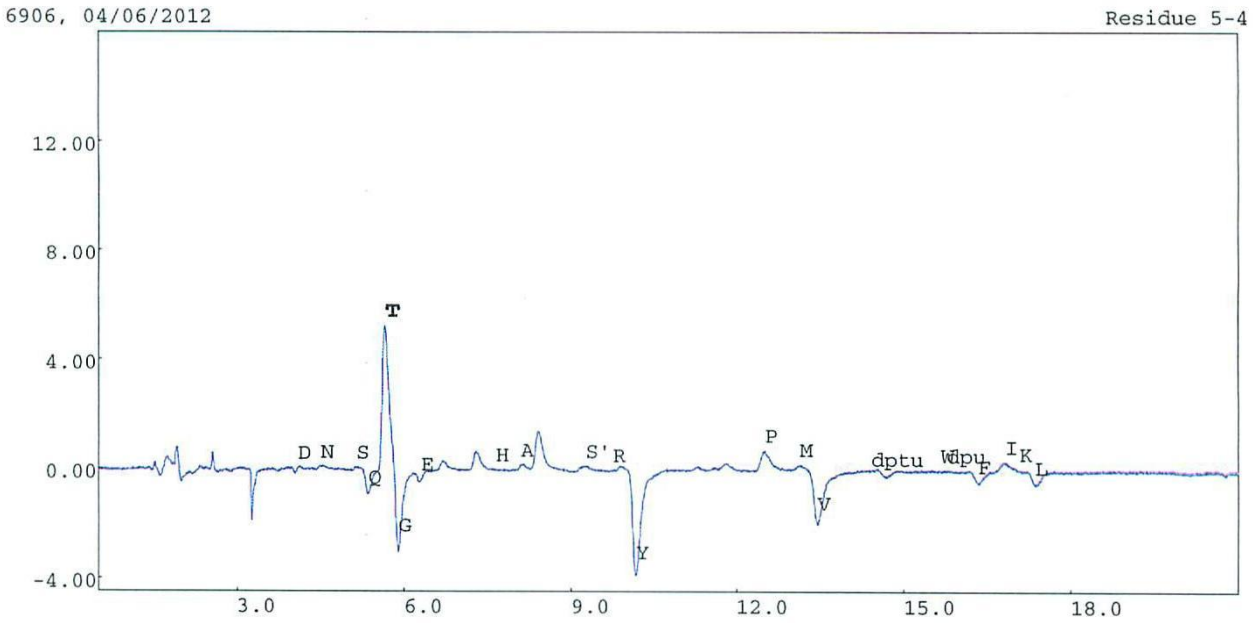


Figure 27 e. Residue 5

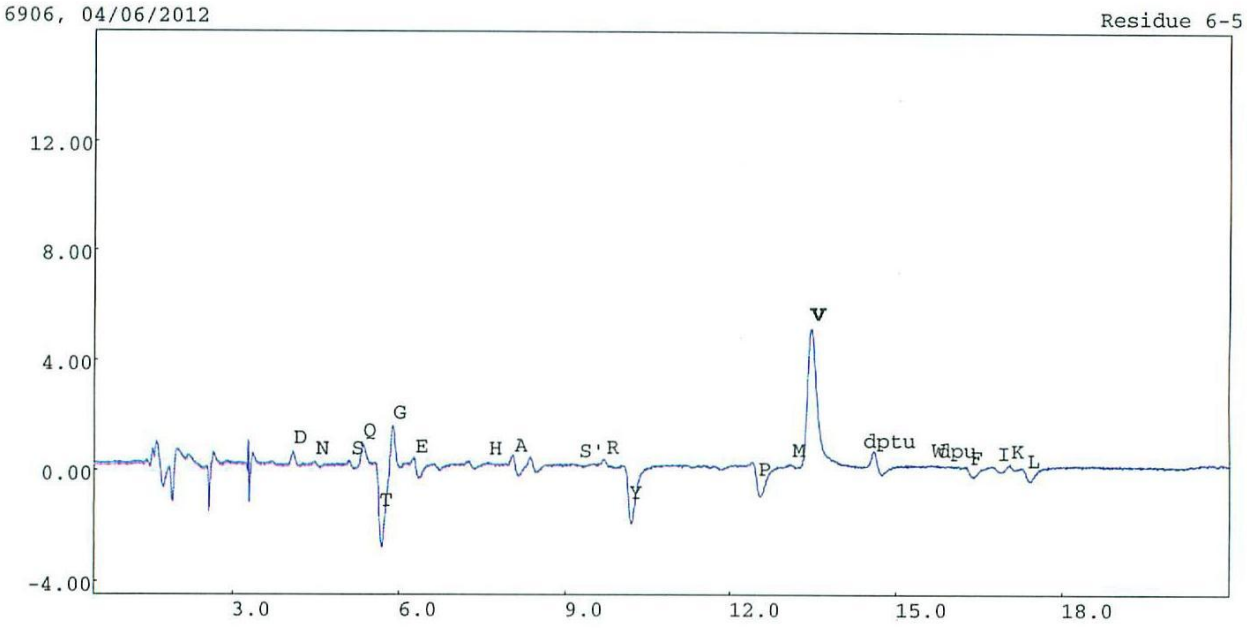


Figure 27 f. Residue 6

4.5.5. Mass Spectrometry of Purified ACP

Two peaks (DIADLQER, VQAMTTMVK and NQQADAQSQIDALESQVSEINTQAQLLAK) were detected in the mass chromatogram; these have been identified as being similar to the secreted antigen Sag A of *E. faecium.* When both the sequences derived were combined and subjected to a similarity search, they were found to be similar to the secreted antigen Sag A/B produced by *E. faecium*. The individual peaks are given in Figure 28a and 28b.

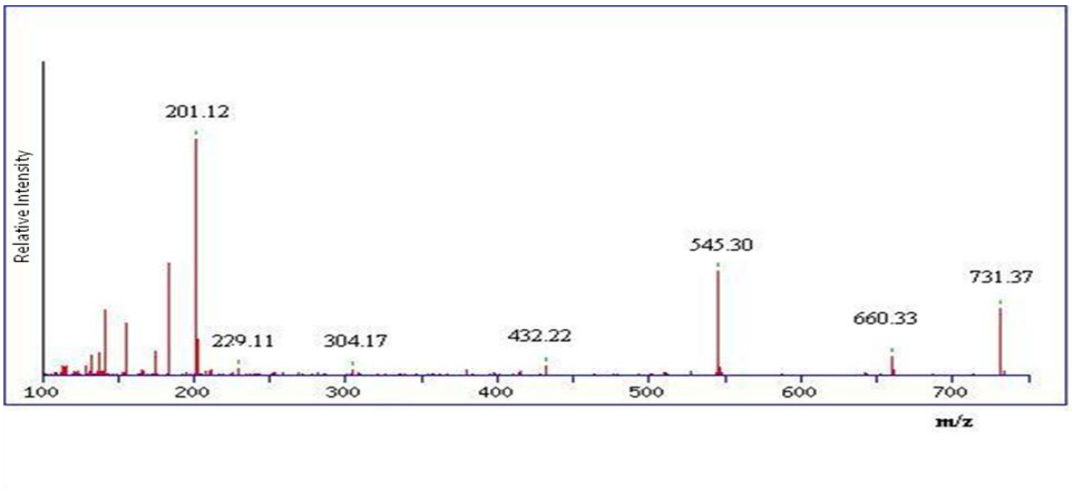


Figure 28a. MALDI-TOF spectra of the ACP, DIADLQER, produced by *E. faecalis*

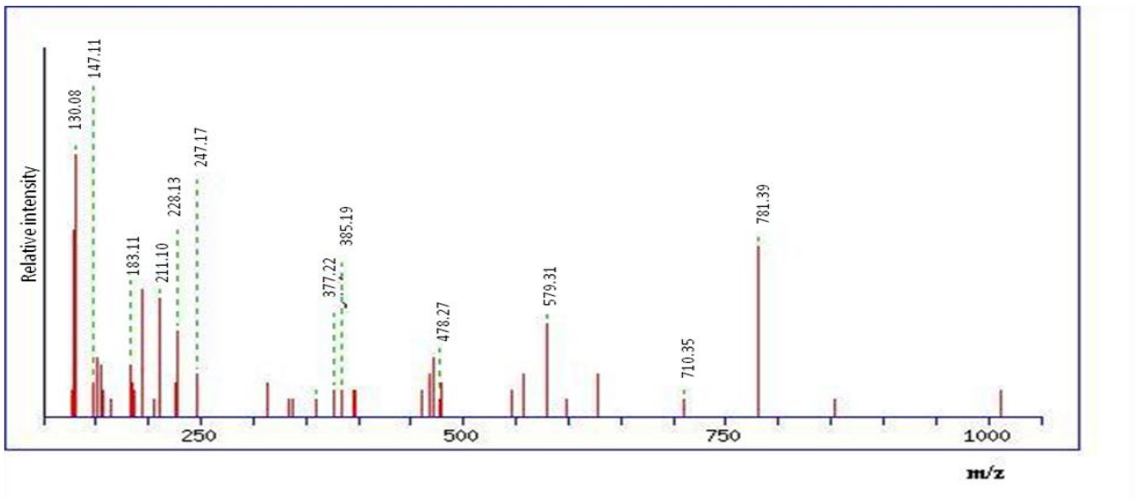


Figure 28b. MALDI-TOF spectra of the ACP VQAMTTMVK, produced by *E. Faecalis*

4.6. Molecular Characterization of ACP

4.6.1. Locus Identification of ACP Genes (Plasmid Curing)

Plasmid curing was observed after treatment with 60 µg/ml of novobiocin (Figure 29), but the biological activity was observed thereafter in the cured sub cultured derivative (Figure 30), suggesting that the antimycotic protein-producing gene might be harboured in the genomic DNA.

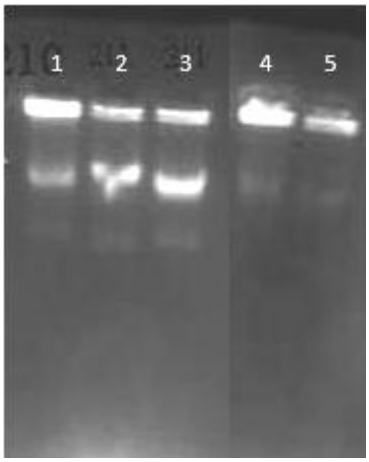


Figure 29. The genomic DNA, native plasmid uncured (lane 1, 2, 3) and 60 µg/ml novobiocin treated culture of *E. faecalis* showing the curing of plasmid (lane 4, 5).

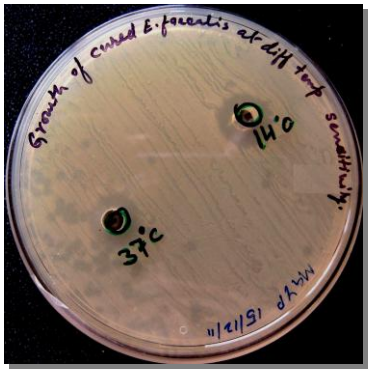


Figure 30. Biological activity of the CFS of cured *E. faecalis* incubated at 14°C and 37°C.

4.6.2. Genomic DNA Library of *E. faecalis*

The genomic DNA was extracted from *E. faecalis* grown in TS broth for 24 hours at 14ºC. For the construction of genomic DNA library, both quantity and quality of DNA are extremely important. The absorbance ratio of the purified genomic DNA at 260/280 nm was 2.16 and the average concentration was 772.5 μg/ml. The isolated DNA was of high quality and good yield with no apparent shearing or degradation.

Genomic DNA was partially digested with *Sau*3A1 and the resulting fragments of size 2 to 5 kbp were separated by agarose gel electrophoresis. The small scale partial genomic DNA digestion was followed by the large scale digestion (Figure 31a). Small as well as large scale complete digestion was also done for pUC 19 vector using *Bam*H1 restriction enzyme (Figure 31b). A library of these fragments was made by ligation to *Bam*HI digested and calf intestinal alkaline phosphatase (CIAP) treated pUC19 and followed by ligation using T4 DNA ligase (Figure 31c). The transformation efficiency was 5.38×10^7 cfu/μg. 1:2 ligation mixtures were plated on 2 plates which gave 213 and 269 white colonies. 2:1 ligation mixture was plated on 2 plates which gave 26 and 53 white colonies. The white clones were randomly selected from the library and checked for the biological activity. Three positive clones named 36C, 38C and 40C were found biologically active against the indicator strains MTCC 183 and MTCC 3958.

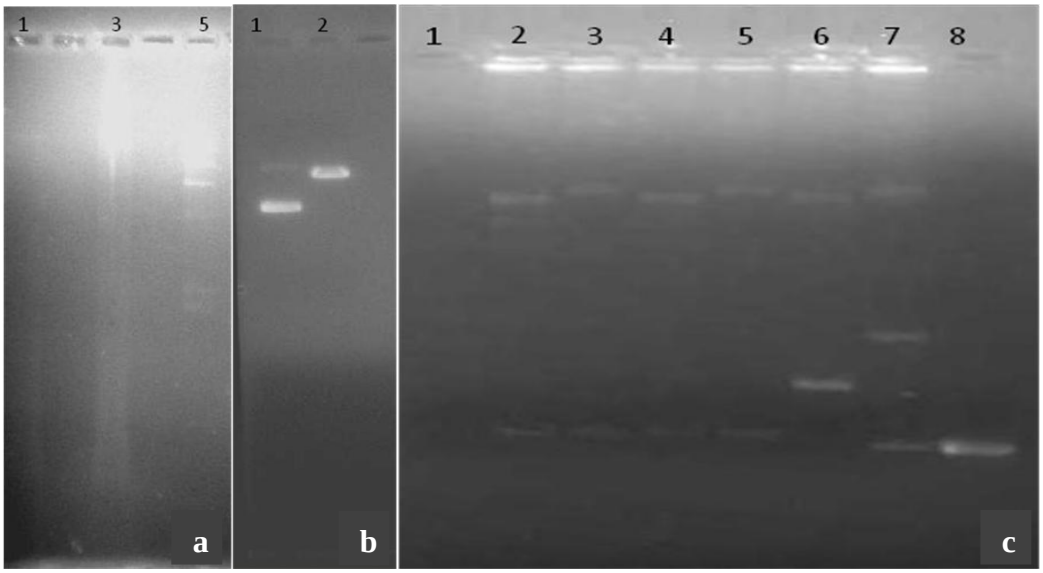


Figure 31. (a) small scale partial digestion of genomic DNA by *Sau*3AI of different concentration, lane 1, 0.5U/µl and lane 3, 0.25U/µl, lane 5 λ*Hind* III digest marker. (b) *Bam*H1 digested pUC 19 vector, lane 1, undigested pUC19, lane 2, digested pUC19. (c) Clones (36C, 38C and 40C) in pUC19 vector. Lane 1, 1kb supercoiled ladder. Lane 2, *Bam*H1 digested clone 36, lane 3, uncut 36C. lane 4, Bam H1 digested 38C. lane 5, uncut 38C, lane 6, digested clone 40, lane 7, uncut clone 40C, lane 8,uncut pUC19 plasmid.

4.6.3. Sequence and Open Reading Frames (ORFs) of Clones

Sequencing of the 36C and 40C clones was achieved successfully (Figure 32a and 32b). Within the sequence region of clone 40C, a 90-bp ORF was identified by the "ORF Finder tool (*www.bioinformatics.org*)" that encodes a protein of 30 amino acids (Figure 33). Database searches using NCBI-BLASTp for antimycotic proteins with similar amino acid sequences indicate that the product of the ORF was similar to the hypothetical protein pBMB0558_00760 of *Bacillus thuringiensis* CT43 (Liu *et al.* 2010), with 93% query coverage, an E-value of 3e-22 and 100% identity. The translational product of the ORF had average homology scores with the lipopeptide antibiotic iturin A produced by *B. subtilis* (Grover *et al.* 2010); only the small patches GH, CF, IR and TYE were matched. Database searches for the hypothetical product of the ORF of clone 36C for relatedness to other known proteins, revealed no significant similarity that could shed light the function of the translated product.

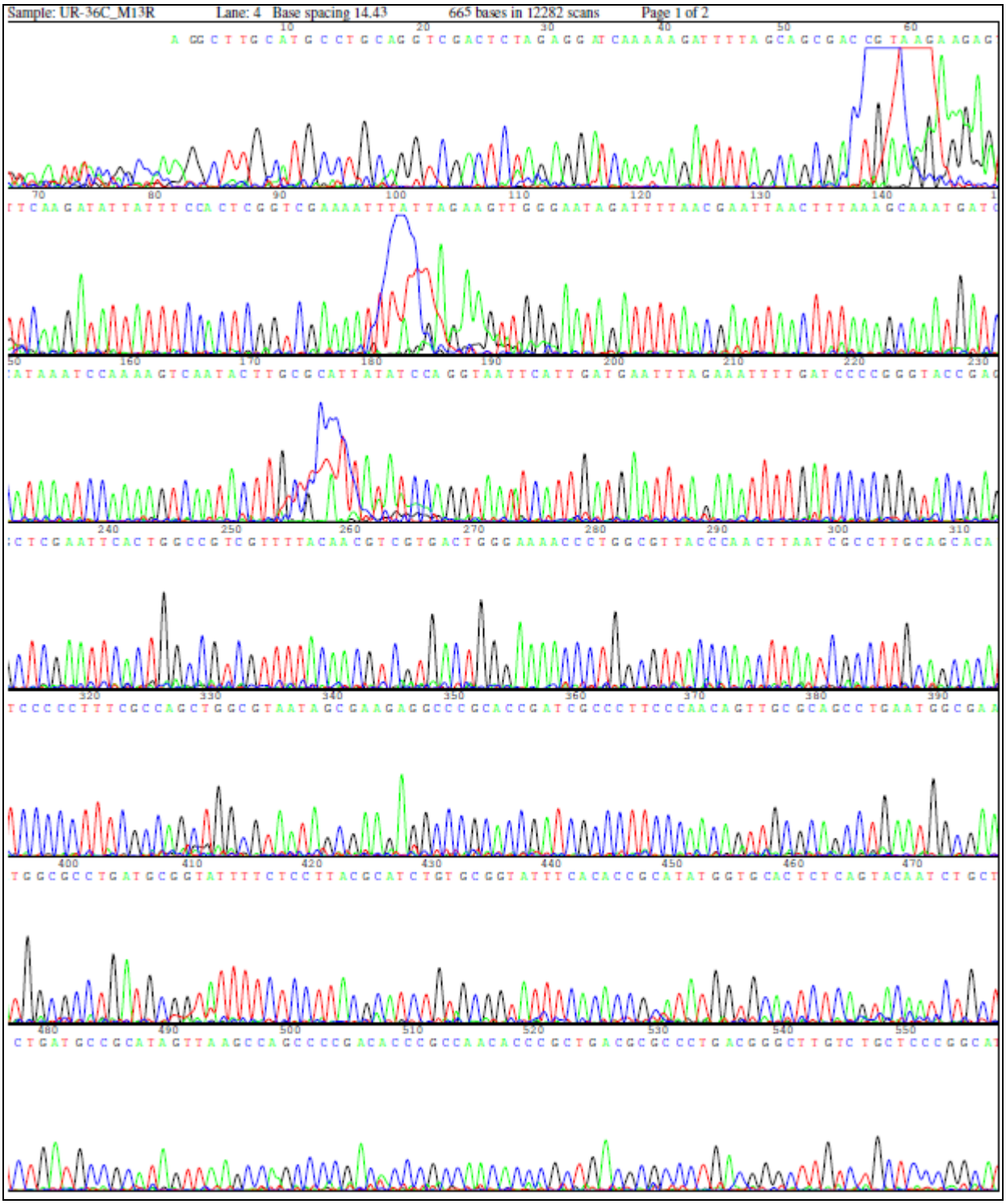


Figure 32 (a).The chromatogram of nucleotide sequence of biologically active clone 36C.

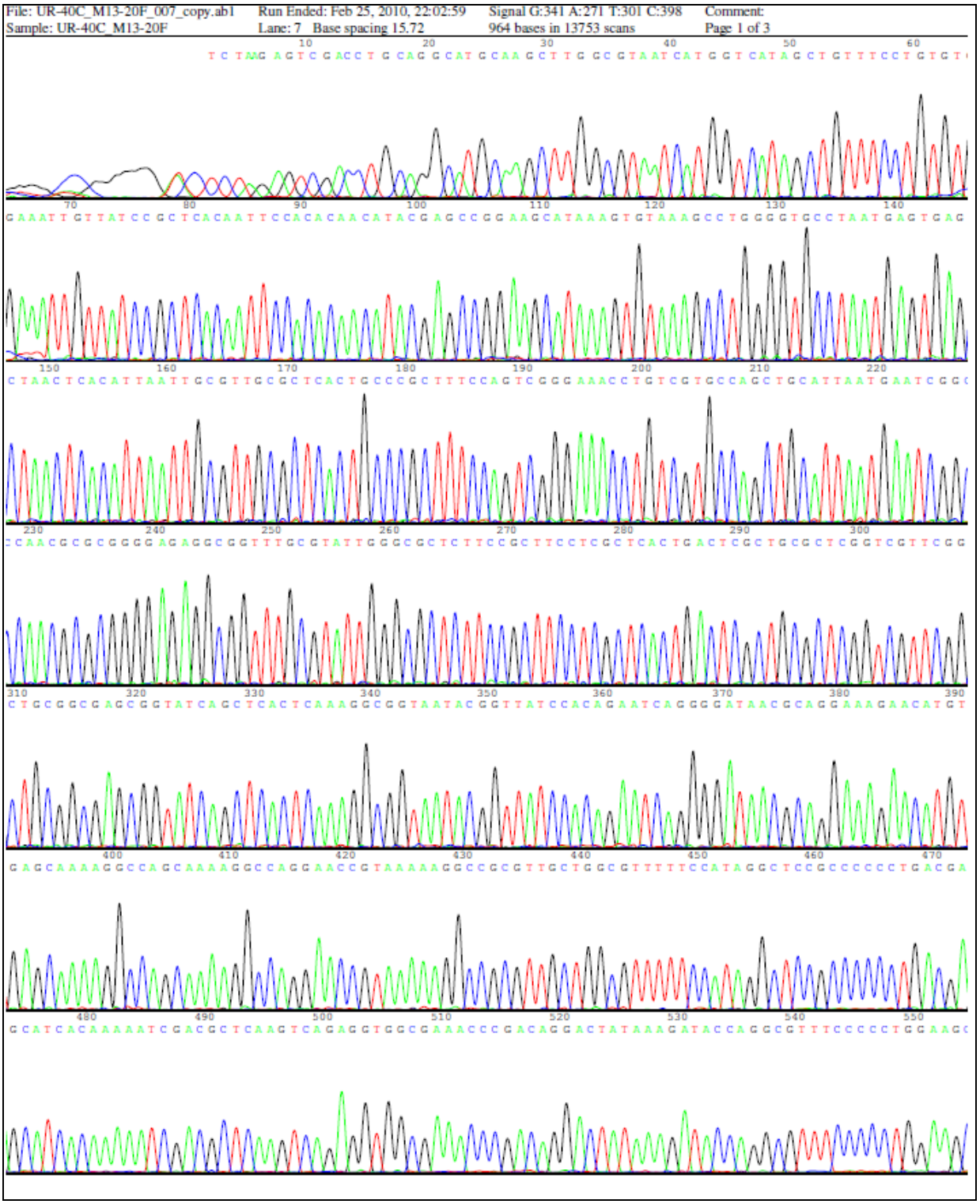


Figure 32 (b).The chromatogram of nucleotide sequence of biologically active clone 40C.

```
1    TCTAAGAGTCGACCTGCAGGCATGCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGT
                ORF Clone 40> M  Q  A  W  R  N  H  G  H  S  C  F  L  C
64   GAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCT
     E  I  V  I  R  S  Q  F  H  T  T  Y  E  P  E  A  *
127  GGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGT
190  CGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTG
252  CGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGC
317  GAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCA
380  GGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGC
442  TGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGA
506  GGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGC
570  GCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTG
635  GCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGG
694  GCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGA
758  GTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAG
821  AGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAG
884  AAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAG
947  CTCTTGATCCGGCAAACA
```

Figure 33.Nucleotide sequence of the 964-bp *Sau*3A1-digested fragment of clone 40C containing the probable antimycotic gene, and the deduced amino acid sequence of the precursor form of the respective antimycotic gene. Amino acids are indicated by their single-letter designations below the nucleotide sequence.

4.6.4. Induction Studies of ACP Genes with Mitomycin C

The potential for induction of inhibitory agents, such as bacteriocins or bacteriophages, lies in the possibility to increase the yield of these agents in bacterial cultures upon treatment with DNA-damaging agents, such as mitomycin C and UV irradiation (Lara *et al.* 1990). In this study mitomycin C induction study was conducted on wild type *E. faecalis* strain. The absorbance values obtained at 4h (Figure 34 a and b) indicate that the cultures which were exposed to different concentration of mitomycin C have less absorbance values than the control, it means the proliferation of cells were affected after mitomycin C addition. For the culture which was exposed to 5 and 10 μg/ml of mitomycin C (for both 30 min and 60 min), absorbance values obtained at different time intervals were maintained at the same range, which indicate that the culture did not recover from the mitomycin C induced damage.

It is clear from the results of cut well agar assay (there is no significant difference in the zone size) (Figure 35a). But when the culture was exposed to 2.0 μg/ml of mitomycin C for 60 min (Figure 35b), absorbance values measured at different time intervals were higher as well as with increased zone of inhibition (ZOI). The results indicated that no induction took place. In an earlier study during mitomycin C induction of *L. lactis* AM2, it was observed that major bacteriolytic enzyme A2 was mitomycin C inducible (Lepeuple *et al*. 1998).

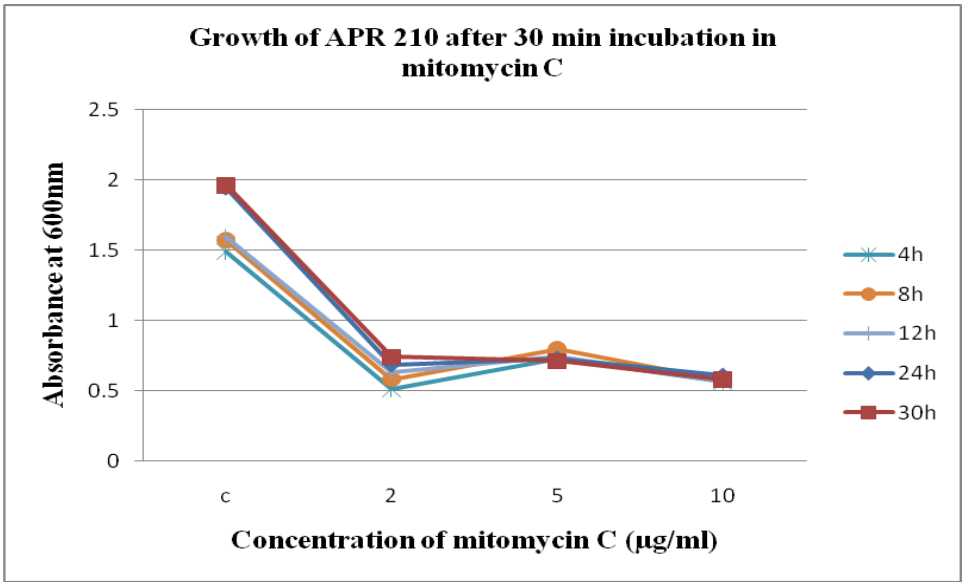


Figure: 34 a. OD values obtained at 600nm of *E. faecalis* cultures those were incubated for 30h with different concentrations of mitomycin C, pre-incubated for 30 mins.

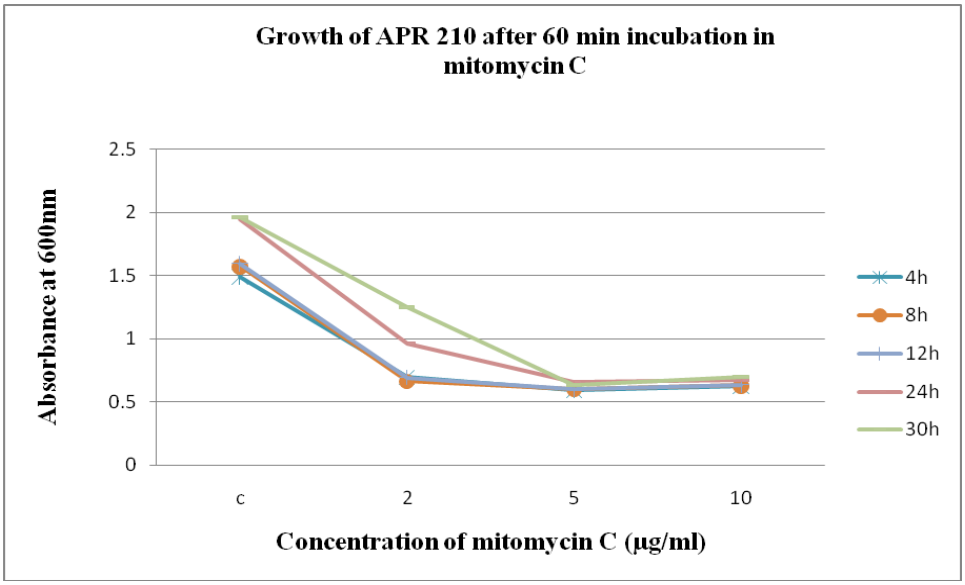


Figure 34 b. OD values obtained at 600nm of *E. faecalis* cultures those were incubated for 30h with different concentrations of mitomycin C, pre-incubated 60 mins.

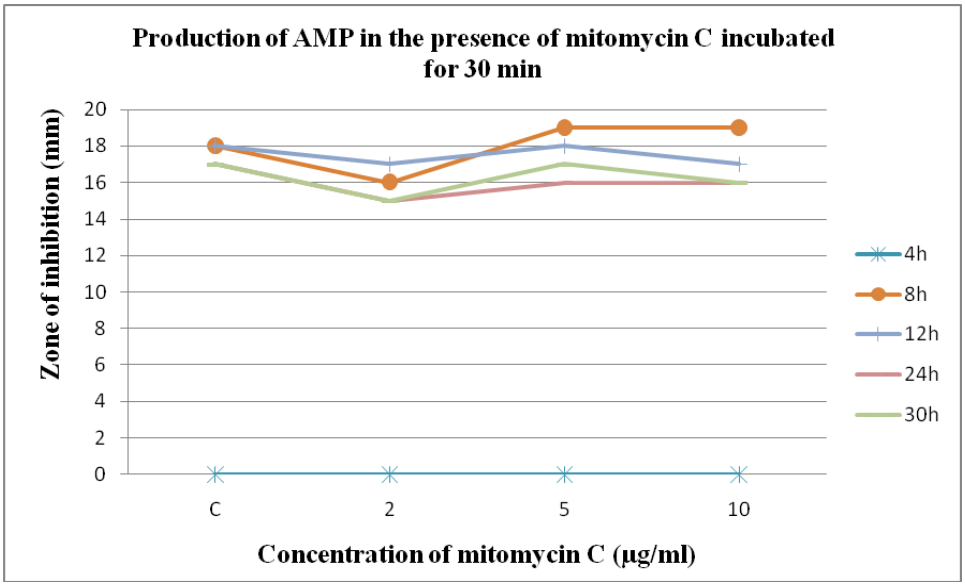


Figure 35 a. Biological activity of CFS in the form of zone of inhibition (ZOI) against *C. albicans* MTCC 3958, pre-incubated for 30 mins with different concentrations of Mitomycin C up to 30 hours.

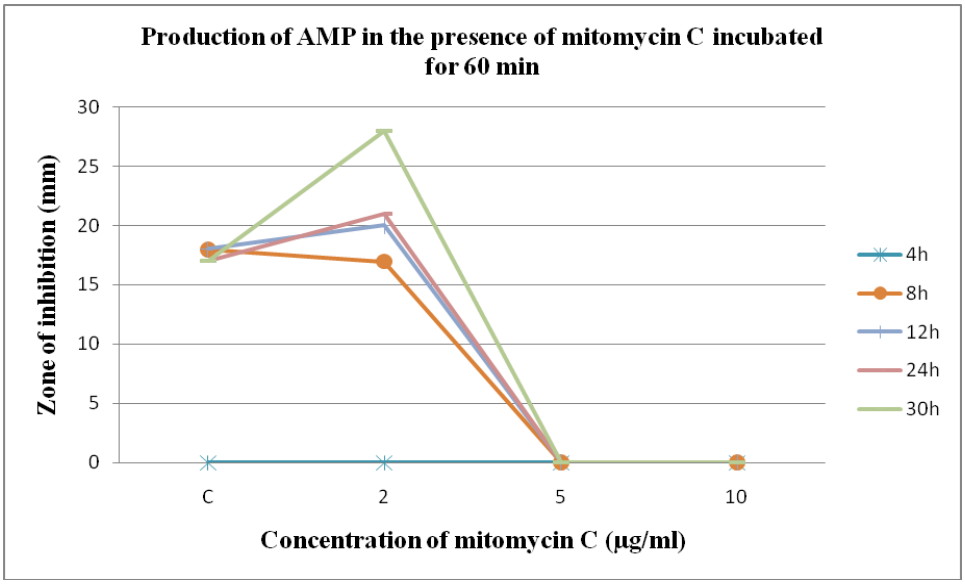


Figure 35 b. Biological activity of CFS in the form of zone of inhibition (ZOI) against *C. albicans* MTCC 3958, pre-incubated for 60 mins with different concentrations of Mitomycin C up to 30 hours.

4.7. Therapeutic Approach of Anti-*Candida* Protein (ACP)

4.7.1. Minimum Inhibitory Concentration (MIC) of ACP against Indicator Organisms

The highest minimal inhibitory concentration (MIC), 1067 µg/ml, of dialysed ACP was found against wild-type *C. albicans* (DI), whereas the lowest MIC, 133µg/ ml, was found against MTCC 183 and MTCC 7315. The MIC of ACP against MTCC 3958 was found to be 267µg/ ml (Shekh *et al*. 2012) (Figure 36).The MIC of DC against MTCC 183 and MTCC 3958 was also checked by spot on lawn method on the plates, spotted different concentrations of DC, that was around 140 µg/ml and 280 µg/ml respectively (Figure 37a, 37b).

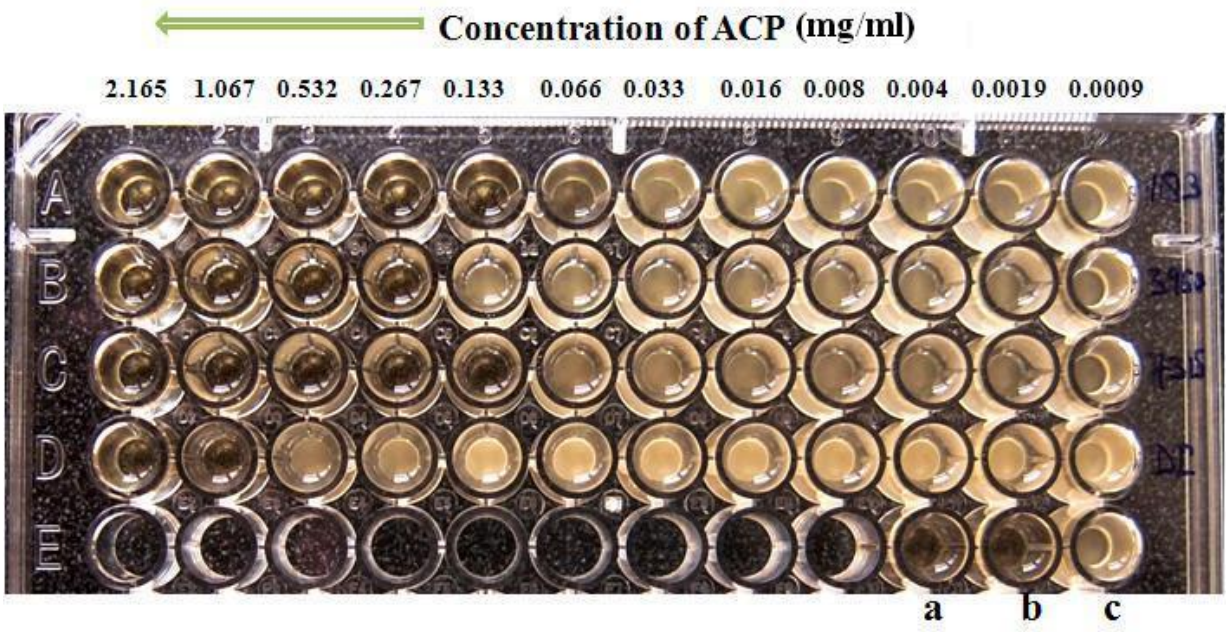


Figure 36. MIC of ACP against *C. albicans* (MTCC 183, MTCC 3958, MTCC 7315, and DI) analyzed by a micro broth dilution assay. Well (a), medium only; well (b), medium containing ACP only; well (c), medium containing grown *C. albicans*. Rows A–D, normal growth of listed strains of *C. albicans;* wells were treated with different concentrations of ACP.

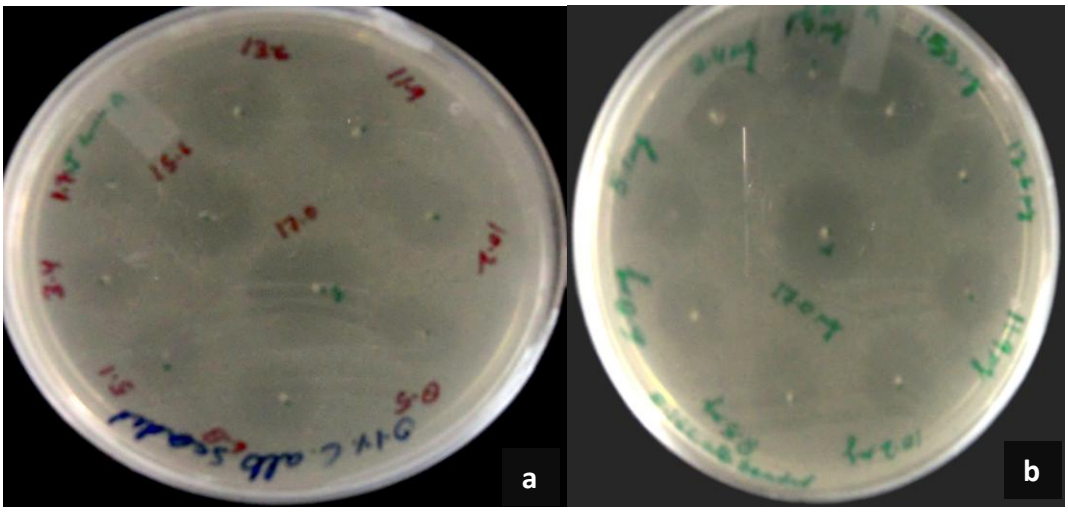


Figure 37a and b. Different dilutions of dialyzed concentrate of anti-Candida protein varying zones of clearance against a lawn of *C. albicans* MTCC 183.

4.7.2. Hemolytic and Haemagglutination Activity Assays

Freshly grown *E. faecalis* APR210, streaked on sheep blood agar plates, did not produce a clear hemolytic zone, indicating that it is a γ-type hemolytic strain; whereas a clear transparent zone was produced by the control, *S. pyogenes* (Figure 38). The cytotoxic effect of the extracellular proteins of *E. faecalis* against human RBCs was determined by hemolytic and haemagglutination assays. The effects of various concentrations of the purified anti-*Candida* compounds on human erythrocytes are reported in Figure 39. The ACP showed negligible haemolytic activity at concentrations up to 0.4 mg/ml, whereas a very weak hemolytic activity (3.76%) was seen at 6.4 mg/ml. The ACP showed no haemagglutination activity at concentrations up to 1.6 mg/ml; however, slight haemagglutination activity was observed at 3.2 mg/ml (Figure 40) (Shekh et al. 2012).

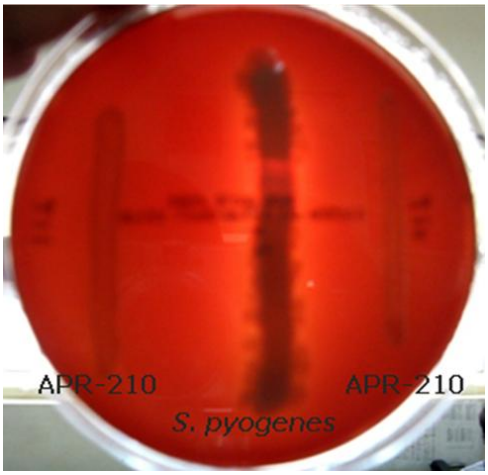


Figure 38. *S. pyogenes* MTCC 442 and *E. faecalis* APR210 streaked on a sheep blood agar plate. A zone of clearance was present in the control (*S. pyogenes*), whereas no clearance was observed in *E. faecalis* APR210, indicating γ-type inhibition.

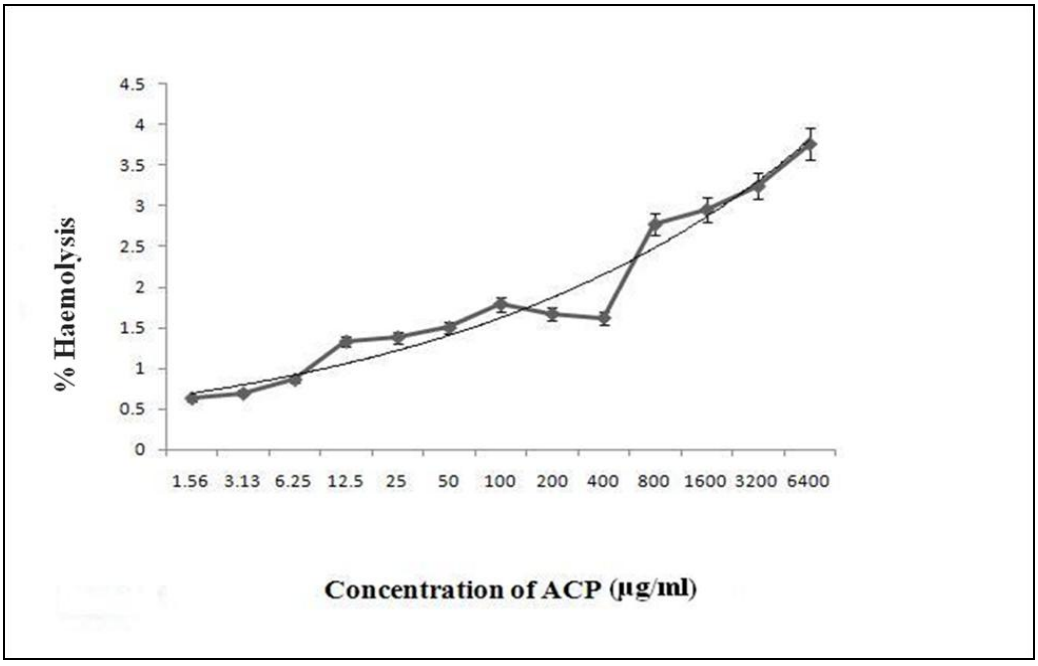


Figure 39. Hemolytic activity of the dialysed ACP concentrate against human erythrocyte cells.

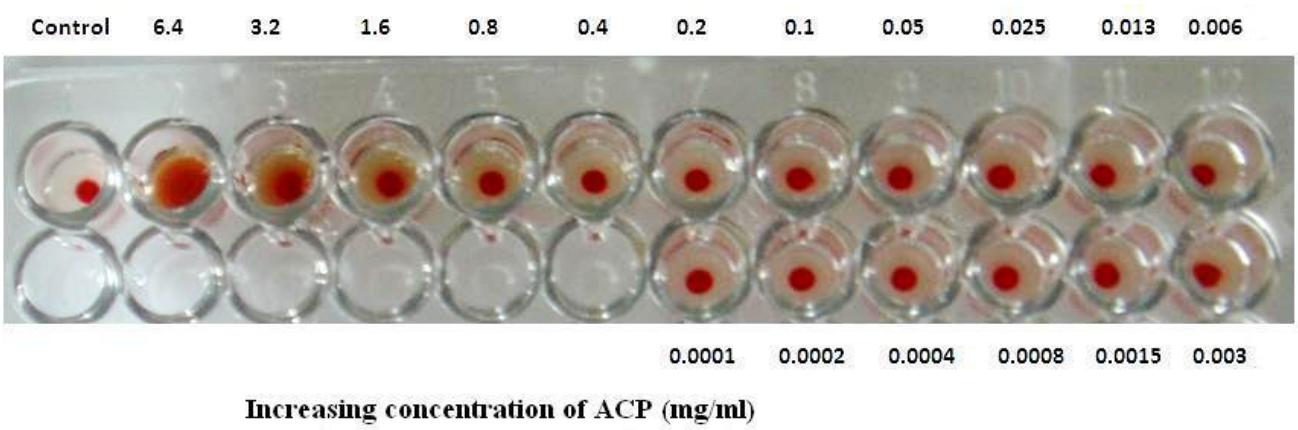


Figure 40. Haemagglutination activity of ACP at different concentrations.

CHAPTER 5

DISCUSSION

Chapter 5 *DISCUSSION*

Biochemical characteristics and fatty acid methyl ester (FAME) analysis identified the most promising strain as *E. feacalis*, whereas 16 S rDNA sequencing identified the strain as *E. faecium* (Shekh *et al*. 2011). Potassium tellurite reduction, however, distinguished the strain as *E. faecalis* rather than *E. faecium*. The concentrate made from the CFS of the test strain inhibited seven multidrug resistant strains of *C. albicans.*

In our study the test strain showed vancomycin resistance. Vancomycin resistance is of particular concern because of treatment difficulties and because of the potential for this plasmid-mediated vancomycin resistance trait to be transferred to other microorganisms (Cetinkaya *et al*. 2000). *Staphylococcus aureus* has been rendered vancomycin-resistant through the apparent transfer of resistance from *E. faecalis* (Huyele *et al*. 1998). A dramatic increase in vancomycin-resistant enterococci (VRE) has been reported in the past years (Cetinkaya *et al*. 2000); from 1989 to 1993, the percentage of nosocominal infections due to VRE reported to the Centers for Disease Control (CDC), increased from 0.3% to 7.9% (Huyele *et al.* 1998 and Mundy *et al*. 2000). It was directly attributed to a rise in VRE infections in an intensive care and non-intensive care unit settings (Huyele *et al*. 1998). *Enterococci* have intrinsic low-level resistance or relative resistance to penicillins, cephalosprins, aminoglysides, and lincosamides (Mundy *et al*. 2000). In recent years, many strains have acquired high-level resistance to multiple antibiotics including aminoglycosides, ampicillin, and vancomycin. Some strains readily acquire resistance to tetracyclines, macrolides, and chloramphenicol.

There are several bacteriocins from *E. faecalis* and other related species (Ennahar *et al*. 2001 and DeVuyst *et al*. 2003), but reports on antimycotic peptides or proteins from *E. faecalis* and *E. faecium* are rare (Roy *et al*. 2009 and Matejuk *et al.* 2010). The genus *Enterococcus* belongs to a group of important lactic acid bacteria (LAB) that participate and contribute towards different fermentation processes. Their functionality in dairy and meat products has been reported in detail (Giraffa *et al*. 2003 and Hugas *et al*. 2003). Several antifungal peptides (iturins, bacillomycins) were discovered from *Bacillus* and *Pseudomonas.* Various strains of *Bacillus subtilis* produce

iturin A and bacillomycin L peptide. Iturins inhibited the growth of fungi including *A. niger*, *C.albicans*, and *F. oxysporum* (Landy *et al*. 1948 and Mhammedi *et al*. 1982). Initial clinical trials involving humans and animals showed that iturin A was effective against dermatomycoses and had a wide spectrum of antifungal properties and low allergenic effects (Billstein *et al*. 1994). Unfortunately, bacillomycin L and iturin A are hemolytic, which may reduce their potential use as antifungal drugs (Latoud *et al*. 1986).

In an era of increased incidence of fungal infections in immunocompromised patients (Ostrosky-Zeichner 2002 and Venkatesan *et al*. 2005) and greater resistance to antifungal therapies (Prasad *et al.* 2005), there is a growing need to discover new antifungal therapies**.**The mechanisms of acquiring multidrug resistance to azole antifungal agents have been elucidated in *Candida* species and can be mainly categorised as (i) changes in the cell wall or plasma membrane that lead to impaired drug (azole) uptake; (ii) alterations in the affinity of the drug target Erg11p (lanosterol 14μ-demethylase) especially to azoles, or in the cellular content of Erg11p due to target site mutation or overexpression of the *ERG11* gene; and (iii) efflux of drugs mediated by membrane transport proteins belonging to the ATP-binding cassette (ABC) transporter family, namely CDR1 and CDR2, or by the major facilitator superfamily (MFS) transporter CaMDR1 (Mishra *et al.* 2007).

Although newer azole derivatives such as voriconazole are more effective and have cidal activity against filamentous fungi such *A. fumigates* (Chandrasekar *et al*. 2000), these derivatives are fungistatic and not fungicidal against pathogenic yeasts. In our study all the indicator strains of *C. albicans* except *C. albicans* (WI) were found sensitive to voriconazole. Amphotericin B has also been commonly used to treat serious fungal infections, but in contrast to azoles, amphotericin B is fungicidal against yeasts. Nevertheless, resistance to amphotericin B is slowly developing in selected *Candida* species (Perea *et al*. 2000) and there are significant side effects associated with its use, including nephrotoxicity. Although recently developed antifungal agents, including the peptide-based agents' micafungin and caspofungin, are very promising, resistance to these therapies has already been reported (Hernandez 2004; Hakki *et al*. 2006 and Thompson *et al*. 2008) and will no doubt become more widespread. The development of resistance to

currently available antifungal agents, the limited efficacy, and the side effects associated with several of these agents increase the importance of continued development of new alternative approaches.

Antimicrobial substances from LAB have been well studied, especially in terms of their antibacterial effect in several forms. However, reports are scanty on the antifungal activity of LAB (Magnusson *et al*. 2001 and Roy *et al*, 2009). The present study systematically attempted to address the candida-related problems by ferreting out a highly potential Gram-positive *E. faecalis* capable of producing an antimycotic substance that demonstrated unequivocally a strong and broad-spectrum anti-Candida activity. The identified *E. faecalis* strain produces the antimycotic principle, ACP, extracellularly. The activity of the ACP was stable upon treatment at different temperatures, for up to 90 °C for 20 min but the activity was completely lost after boiling and autoclaving. While similar results have been reported for durancin L28-1A from *E. durans* (Yanagida *et al*. 2005) and bacillomycin D from *B. subtilis* (Tabbene *et al*. 2011), bacteriocin ST15 from *E. faecium* was found inactivated when subjected to 121 °C for 20 min (De Kwaadsteniet *et al*. 2005). The antimycotic property of the ACP in the present study also remained unaffected in the pH range of 6.0–8.0. At pH values of 5.0 and 9.0, however, the activity was reduced by 50% whereas at values of pH 2.0, 4.0 and 10.0 activity was completely lost. These results are in agreement to those reported for the bacteriocin produced by *E. mundtii* (Ferreira *et al*. 2007). Several bacteriocins produced by *Enterococci* are known to exhibit a wide range of pH stability (Losteinkit *et al*. 2001). The ACP was stable when treated by different organic solvents and surfactants; such stability has been a common feature of many bacteriocins produced by *E. faecalis,* AMPs produced by *Bacillus* species, and other LAB (Atrih *et al*. 2001; De Kwaadsteniet *et al*. 2005 and Hernandez *et al*. 2005).

Optimization of growth parameters revealed that the maximum ACP was produced in modified Tripticase Soya Broth (mTSB) at the pH range of 7.0-8.0 and 15º- 35°C after 48 hrs of incubation with 1.0% inoculum. The activity was lost after 120 hrs. The moderate increase in ACP production was noticed at 2.0 - 5.0% NaCl. In case of *E. faecium* CTC492 it was recorded that growth was faster at higher pH values. The maximum biomass was reached after 18hr at pH

8.0, compared to 34.5 hr at pH 6.2, but only about 5% of the optimal bacteriocin yield was obtained at this high pH. When *E. faecium* CTC492 was grown at pH 5.5, growth was very slow and bacteriocin production was low (Nilsen *et al.*1998). In another study, Enterocin P was produced in MRS broth from 16 to 45°C (Cintas *et al*. 1997). In case of *L. lactis* isolated from marine environment maximum bacteriocin production was observed at 30°C, pH 6.0 and 1.5% sodium chloride solution (Rajaram *et al.* 2010)

In the proteolytic study, the ACP was found to be fully sensitive to proteinase K and partially sensitive to pronase E, confirming its proteinaceous nature (Shekh *et al*. 2011; Shekh and Roy 2012). Its resistance to pepsin and trypsin indicated that the anti- *Candida* active principle may be a cyclic peptide containing unusual amino acids and therefore more resistant to protease hydrolysis (Bizani *et al.* 2002).These results suggested that this antimycotic peptide could survive in the intestinal environment and might therefore be administered with food (Jianhua *et al*. 2009).

On the other hand, the ineffectiveness of α-amylase and lipase on antimycotic activity suggested that the ACP might not be glycosylated and might not contain a lipid moiety. When the ACP was heated with 1 mM and 2 mM β-mercaptoethaol at 80°C for 10 min to ensure thiol residues existed in the reduced state, no particular change in antimycotic activity was observed. This indicates that the oxidation state of the cysteine residues may not be important for the antimycotic activity (Hastings *et al*. 1991). Magnusson *et al.* (2003) reported that a proteinaceous compound produced by *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 had antifungal effect against several moulds and against the yeasts *Debaromyces hansenii* and *Kluyveromyces marxianus*. The peptide was small (approximately 3kDa), heat stable active in the pH range 3-6 and totally inactivated by proteinase K or trypsin. The same characteristics could be found among bacteriocins of subclass II (Nes *et al.* 2000). In our study when the dialysed concentrate, was treated with the reducing agent DTT, no decrease in inhibitory activity was observed, indicating that disulphide bonds are not responsible for biological activity. It was also observed that storage of ACP at −80 °C for 1 year did not significantly affect the biological activity.

Ammonium sulfate salt as well as sodium phosphate buffer did not inhibit ACP activity at the concentration used and did not modify the results of the assay. The dialysed concentrate of ACP, dissolved in 20 mM sodium phosphate buffer, weakly bound with the DEAE Sepharose matrix, indicating that the ACP bears negative charges only at the buffer range of pI. Being weakly negative, it was separated easily in native polyacrylamide gel electrophoresis. After purification by ammonium sulfate fractionation, dialysis, anion exchange chromatography and gel filtration, the final amount of recovered protein (0.45 %) was found very low. This could be increased by using optimization methods and other protein engineering methods.

Comparing the partial amino acid sequence of the purified antimycotic protein to other antimicrobial peptides and bacteriocins by using protein-protein BLAST in NCBI revealed no complete homology with other known bacteriocins or AMPs. The combined N-terminal and *de novo* sequence GPGGPG…WLPPAGLLGRCGRWFRPWLLWLQSGAQYKWLGNLFGLGPK had high amounts of glycine, proline, leucine and tryptophan. This has been observed in many antimicrobial peptides including bacteriocins like enterocin and acidocin (Shekh and Roy 2012).

It was reported earlier that the glycine-rich antifungal peptide tenacin-3 enters the *C. albicans* cytoplasm (Kim *et al*. 2001), although tenacin-3 seems not to induce membrane permeabilisation. Linear peptides with an extended structure were characterised by an unusual proportion of one or more amino acids (most often proline, tryptophan, or glycine (Bulet *et al*. 1991 and Otero-Gonzalez *et al*. 2010). Penaedins characterised from shrimps and prawns had a high content of Pro/ Arg/ Gly residues in the extended N-terminal domain (Rodriguez *et al*. 2011). Oxypinin 2 has a GVG motif, and ponericin G has glycine residues flanking the central proline, resulting in a GPG motif with calculated grand average of hydropathicity (GRAVY) of −0.683.20. The presence of Gly-Pro hinges in antimicrobial peptides like oxypinins, ponericins, and cecropins supports the antimicrobial potential of ACP, wherein a similar motif was observed (Shekh and Roy 2012). The regional flexibility provided by proline was sometimes enhanced by the presence of glycine residues (Cordes *et al*. 2002). In another recent report, a penaedin homologue, hyastatin from spider crab (Capinera 2008), was shown to possess a Pro/Gly domain similar to the N-terminal domain of penaedins that bind chitin tightly. This information

strengthens the idea that the N-terminal minor sequence GPGGPG of the anti-*Candida* protein in the present study could interact with the cell wall of *Candida* as a primer for antimicrobial action (Capinera 2008). In such a proline-rich sequence, a proline kink has all the potential to create pores (Dempsey *et al*. 1991). As was mentioned in the earlier section complete homology was not found in the existing protein database. However, the GPGG sequence was found to match with that of a known ABC transporter, ABC transporter peptide permease, and a hypothetical protein. The first 3 amino acid residues, GPG, matched the N-terminal sequence of enterocin 1071B (Balla *et al*. 2000 and Franz *et al*. 2002). Likewise, the GPG sequence was also observed in EntC2 (Maldonado-Barragan *et al*. 2009).

It was cogently argued that in cationic hydrophobic peptides the presence of polar residues confers a hydrophilic property to the proline-rich peptides. In an earlier study conducted on curvaticin FS47, the neutral (Gly, 24%) and hydrophobic (Ala, Ile, Leu, Val, Pro, and Phe, 47%) residues at the N-terminal constitute a significant proportion which helps to explain the hydrophobic interactions that curvaticin FS47 displays (Garver and Muriana 1994). It was reasoned that the high proportion of Gly residues (23.9% in ACP) would likely provide a significant amount of flexibility to the antimicrobial molecule (Garver and Muriana 1994). In fact, the increase of hydrophobicity of the peptides also correlated with fungicidal activity (Lee *et al*. 2002). In accordance with many other bacteriocins of LAB e.g., lactococcin A (Holo *et al*. 1991), lactacin F (Muriana *et al*. 1991), and curvaticin FS47 (Garver *et al*. 1994), a high proportion of glycine was likely to provide a significant amount of flexibility to the molecule. A recent study on lactococcin G, enterocin 1071B, and EntC2 suggested that the N-terminal sequence of the peptide of each bacteriocin (LcnGβ, Ent1071B and EntC2) is important for determining target cell specificity (Maldonado-Barragan *et al*. 2009 and Oppegard *et al*. 2007). Analysis of the major N-terminal sequence DEVYTVKS(S+S´) GDSL revealed the presence of S´, suggesting that serine is modified; this is a feature of class I antibiotics (Begley *et al.* 2009). This sequence was found to be very similar to that of autolysin and a hypothetical protein of *E. faecalis* in BLASTp search.

Previously, the N- terminal sequence of the antimicrobial dermaseptin B was reported to be highly hydrophobic which could enable its binding to zwitterionic outer and negatively charged

surfaces (Shai 2002). In addition, the part of the N-terminal sequence which contains Gly- Pro residues and the combined de novo sequence detected in the anti-*Candida* protein ACP under current investigation, were supported by the inference that proline-rich peptides (often associated with arginine) enter cells without membrane lysis and after entering the cytoplasm bind to and inhibit the activity of specific molecular targets causing cell death (Gennaro *et al*. 2002).

Other studies with model amphipathic all L-amino acid peptides with the sequence KX3KWX2KX2K, where X = Gly, Ala, Val, or Leu, showed that the leucine-rich peptide, rather than the Ile- or Val-containing peptide, was particularly antimicrobial (Shai, 2002). Our result is in partial agreement with this observation: leucine amounted to 19.6%, and proline (13.0%) was found in association with arginine. The combined sequence derived from *de novo* sequencing, WLPPAGLLGRCGRWFRP...WLLWLQSGAQY....KWLGNLFGLGPK, showed high glycine (17.5%), proline, leucine, and tryptophan content (Shekh and Roy 2012). The amino acid content also revealed that the peptide was quite hydrophobic due to the presence of high amounts of leucine (22.5%); this is believed to play a role in its interactions with the cell membrane (Muriana *et al*. 1991). The hydrophobicities (GRAVY) of individual peptides with *m/z* values of 718, 1039, and 601 were 0.108, -0.388, and 0.282 respectively, indicating that these peptides are relatively hydrophobic; this is characteristic of many bacteriocins isolated from *Enterococcus* species (Cintas *et al*. 1998). High levels of glycine (31%) and glutamine (18%) residues in another cationic antifungal peptide constitutively produced by *S. peregrine* larvae were also reported to bind *C. albicans* through electrostatic interactions and disturb the osmotic integrity of treated cells (Capinera, 2008). In contrast, a novel glycine/leucine-rich antimicrobial peptide, leptoglycine (59.1% glycine and 36.4% leucine), derived from *Leptodactylus pentadactylus* failed to inhibit *C. albicans*.

The minimum inhibitory concentration (MIC) of the ACP against wild-type *C. albicans* DI was 1067µg/ml, whereas the lowest MIC recorded, 133µg/ml, was against MTCC 183 and MTCC 7315 (Figure 36). The MIC of the ACP against MTCC 3958 was 267µg/ml that is slightly higher than the MICs of iturin and bafilomycin F (Mhammedi *et al*. 1984).

In the present study, it was attempted to induce the gene of interest. Mitomycin C induction was attempted with different concentrations at different time intervals. The result from the figures 35a and 35b indicated that no induction took place. This observation is the indication of the recovery of the culture from the damage. If the bacteria were lysogenic, the recovery could not have occurred; the phage induction if occurred could have completely lysed the cells. Nakagawa (1979) was also unable to induce bacteriocin production in three bacteriocinogenic strains of *S. faecalis* and one strain of *S. faecium* upon mitomycin C treatment or UV irradiation. Simpson and Tagg (1983) also described streptoccin A-M57, produced by the group A streptococci type M-57, as non inducible by the above treatments. However, Lara *et al*. (1990) was successful in inducing the production of bacteriocin Bc-48 in the mutant B-48-28 of *E. faecalis* ssp. *liquefaciens*.In an earlier study during mitomycin C induction of *L. lactis* AM2, it was observed that major bacteriolytic enzyme A2 was mitomycin C inducible (Lepeuple *et al*. 1998).This suggested that it was encoded by prophage DNA (Lepeuple *et al*. 1998). However in another experiment conducted later, mitomycin C was added to exponentially growing NCC 533 cells at a concentration known to induce prophages in other lactic acid bacteria (Brussow *et al*. 1995).
In an earlier study of the mitomycin C induction of *L. lactis* AM2, the major bacteriolytic enzyme A2 was observed to be mitomycin C inducible. This suggested that it was encoded by prophage DNA (Anne-Sophie *et al*. 1998). However, in a subsequent experiment, mitomycin C was added to exponentially growing NCC 533 cells at a concentration known to induce prophages in other lactic acid bacteria (Brussow *et al*. 1995). Analysis of the PCR products suggested that mitomycin C induction neither resulted in lysis of the lysogenic cells nor in cell growth. A similar observation was made in another experiment, where treatment with 2 μg/ml mitomycin C did not cause lysis and recovery from the damage was observed in terms of increased OD and enhanced zone of inhibition (Ventura *et al*. 2004).

In the present study the genomic DNA library was constructed to clone an anti-*Candida* protein (ACP) gene by deploying the vector pUC19. The plasmid curing results showed that the gene of interest was harboured in the genomic DNA, because the *E. faecalis* strain shows a reduced plasmid copy number. Analysis of the 964 bp nucleotide sequence revealed that the cloned fragment in clone 40C contained an ORF that encodes a 3300 Da protein of the amino acid

residues MQAWRNHGHSCFLCEIVIRSQFHTTYEPEA. Database searches with NCBI-BLASTp, for proteins with similar amino acid sequences, indicate that the product of the ORF was similar to the hypothetical protein pBMB0558_00760 of *B. thuringiensis* CT43 (Liu, 2010), with 93% query coverage, an E-value of 3e-22, and 100% identity. This hypothetical protein pBMB0558_00760 of *B. thuringiensis* CT43 is reported to possess insecticidal properties. The product of the ORF had an average homology scores with the lipopeptide antibiotic iturin A produced by *B. subtilis* (Grover *et al.* 2008); the small patches GH, CF, IR, and TYE were matched. The encoded protein product of the ORF of the clone 36C (MLRRGQALLVVSGHTSRSSGRASPDVNSPSSHWELRTQTGSLGYCIGGGKLCWWLLT ACRSR) could not be identified.

Previously, an attempt was made to clone and express the bacteriocin helveticin J by constructing a genomic DNA library. The cloned fragment containing an ORF3 was analysed by searching the database for proteins with similar amino acid sequences; however, similar bacteriocins were not found (Joerger and Klaenhammer 1990). Rather, the ice nucleation protein produced by *P. syringae* had the highest homology scores to the predicted product of ORF3 (Green and Warren 1985). However, the presence of 48% of the total glycines in the bacteriocin within the first 100 amino acids is reminiscent of the presence of high percentages of glycines at the N-terminal ends of a number of colicins. This led to the prediction of β-turns and β-sheets at the amino-terminal ends, which are thought to be involved in the uptake of colicin molecules into the target cell (Joerger and Klaenhammer, 1990). Future efforts to establish the function of the ORF-encoded protein product and the completion of the DNA sequence analysis will produce more information concerning the antifungal activity of the protein produced by *E. faecalis*. Such data will facilitate the cloning of the specific antimycotic genes as well as improve the yield.

In LC-ESI-MS/MS, the peptide sequence did not completely match any protein present in the MASCOT database. However, three significant peptides DIADLQER, VQAMTTMVK and NQQADAQSQIDALESQVSEINTQAQDLLAK matched with the secreted antigens Sag A/B produced by *E. faecium* (Holmes *et al*. 1998). The last 30 amino acid peptide sequence having the score 95.9 in LudwigNR, tr|C9B9N8|Secreted antigen SagA Tax_Id=565658 [*E. faecium*].

Extracellular *E. faecium* SagA protein, which is antigenic in nature, is apparently essential for growth and shows broad-spectrum binding to extracellular matrix (ECM) proteins, forming oligomers, whereas the secreted proteins sspA and sspB produced by *L. lactis* adhere to collagen type I and *C. albicans* (Teng *et al*. 2003). In a study by Holmes *et al.* in 1998, the gene in clones d1-27 and d2-29 was named *sagA* for secreted antigen, and was found in all 11 *E. faecium* strains from different clinical and/or community and geographic sources. Taken together, all these data probably indicate that the antimicrobial activity of this ACP is because of some peptides important for binding the indicator organism's cell membrane.

In this study, the results of toxicity experiments were of great interest. ACP was non-toxic to human erythrocytes up to a tested concentration of 6.4 mg/ml (Figure 39). At this concentration, the percent hemolytic activity was 3.76 comparatively much lower than those of baciamin (Wong *et al*. 2008) and bafilomycin F (Mhammedi *et al*. 1982). It was also concluded that ACP did not cause haemagglutination of human red blood cells at concentrations up to 1.6 mg/ml (Figure 40). Higher concentrations did cause haemagglutination of human RBCs; however, this concentration is much higher than the MIC of the ACP. These properties, taken together, probably mean that this anti-*Candida* protein ACP is a potent candidate for treating candidiasis, and its pharmaceutical application can be established in synergy with other relevant low-dose antifungal antibiotics.

CHAPTER 6

SUMMARY, CONCLUSIONS AND FUTURE SCOPES

SUMMARY AND CONCLUSIONS

1. As many as 240 Antarctic and Arctic isolates were screened for production of antibacterial and antimycotic substances. Three isolates AGM 108-5, AGM 111 and APR 210 based on Fatty Acid Methyl Ester (FAME) and 16S rDNA sequencing were identified as *Y. aldovae, C. maltaromaticum* and *E. faecalis*/*faecium* showed strong antimicrobial activity against an indicator multidrug resistant *C. albicans* NCIM 3471 strain in the beginning. After that the cell free supernatant as well as concentrate of *E. faecalis* APR 210 showed strong anti-*Candida* activity against different strains.

2. The anti-*Candida* substance (ACP) was partially hydrolyzed by Pronase E and completely by Proteinase K, suggesting its proteinaceous nature. The ACP was resistant to trypsin, pepsin, α-amylase and lipase. The ACP retained its biological activity with the treatment of 90°C for 20 min. The antimicrobial activity was lost when boiled at 100°C. The activity was retained at the pH values of 6.0, 7.0 and 8.0. However the activity was reduced by 50% at pH 5.0 and 9.0. The ACP was stable in different organic solvents and surfactants.

3. Optimization of growth parameters revealed the maximum ACP was produced at pH 7.0 and at 15º- 35°C after 48 hrs of incubation with 1% inoculum. The activity was lost after 120 hrs. 2.0-5.0% NaCl increases ACP production.

4. The ACP was partially purified by ammonium sulfate fractionation, ion exchange and gel filtration chromatography. The specific activity was increased after each step having the final purification factor 22.4 and recovery 0.45%.

5. The ACP activity was also seen onto Tricine gel containing 10% resolving and 5.0% stacking gel against *C. albicans* MTCC 3958.

6. The antimicrobial activity of the *E. faecalis* strain was found exclusively in the extracellular filtrate produced in the late logarithmic growth phase. The highest activity

(1600 AU/ml) against *C. albicans* MTCC 183 was recorded at 48 h of incubation, and activity decreased thereafter.

7. Partially purified ACP had a molecular weight of about ~ 43 KDa in Tricine-Native PAGE analysis.

8. Safety investigation having haemagglutination, hemolytic activity and antibiotics susceptibility of *E. faecalis* showed non pathogenic characteristics.

9. The MIC of dialysed concentrate ACP was found 1067 µg/ml against wild type *C. albicans* (DI) whereas against MTCC 183 and MTCC 7315 that was 133 µg/ml. The MIC value of DC against MTCC 3958 was 267 µg /ml.

10. The N-terminal amino acid sequence of 12 amino acids residues were obtained by Edman degradation. The major sequence DEVYTVKSGDSL and the minor sequence GPGGPGKS'GDS'L were found. The peptide was *de novo* sequenced by ESI-MS, and the deduced combined sequence when compared with other bacteriocins and antimicrobial peptides had no significant sequence similarity.

11. Plasmid curing experiment suggests that the antimycotic protein producing gene might be harbored in genomic DNA and was not found induced by mitomycin C.

12. A genomic library was constructed in *E. coli* DH5α - pUC19 host vector system. The transformed host *E. coli* DH5*α* harboring a *BamHI* insert in pUC 19 showed clear zone of inhibition against *C. albicans* MTCC 3958. The inserts of interest in two positive clones were sequenced. The nucleotide sequence of the clone 40C revealed an open reading frame (ORF) that has maximum similarity with a hypothetical protein pBMB0558_00760 of *B. thuringiensis* CT43 and average homology scores with lipopeptide antibiotic iturin A produced by *B. subtilis.*

Specific Contributions

1. The properties of this Anti-*Candida* protein based on the data, probably mean that this anti-*Candida* protein ACP is a potent candidate for treating candidiasis, and its pharmaceutical application can be established in synergy with other relevant low-dose antifungal antibiotics.

2. The modes of action of this protein/peptide might be different from the currently used therapeutics, since it is active on several multidrug resistant *Candida* strains, which is becoming a clinical problem.

Future Scopes

1. This study has shown that bacteria recovered from the Antarctic eco-habitat could be the source of potential antimicrobial compound, which may be examined for its suitability in food, feed and therapeutic applications. This anti-*Candida* protein is to be tested for its possible clinical use. This is rather heartening since the modes of action of these proteins are different from the currently used therapeutics, resistance to which is becoming a clinical problem.

2. An understanding of the genetic organization and regulation of *E. faecium* antimicrobial genes will facilitate engineering novel protein antimicrobial agents for therapeutic approach. In this study the total recovery percent of ACP was less that can be enhanced by using some protein engineering or recombinant technology.

3. The chemical synthesis of this compound can be suggested and the costs of this using a solid-phase approach would probably be inexpensive. This approach would benefit a developing country like India to organize the production of this antimycotic peptide.

4. Heterologous expression of anti-*Candida* protein in different hosts especially in suitable *L. lactic* and *E. coli* is desired to be attempted. It is accepted that the use of AMPs would be safe for the treatment of infectious diseases owing to the fact that they are elaborated by probiotics (Fasano et al. 2009).However, systematic investigations are further required to explore the possibility of preparing purified anti-*Candida* peptide without any adverse effect and with a permissible MIC that can pass this compound through pre-clinical studies in preparation for entry into clinical trials.

5. The clinical problems caused by the colonization of the *C. albicans* like yeasts on the hospital equipment and human blood with the help of such kind of antimicrobial protein can be studied.

6. The antimicrobial compound may have potential application in the treatment of cancer.

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Appendix- i

LIST OF PUBLICATIONS

1. **Raeesh M. Shekh** and Utpal Roy (2012) Biochemical characterization of an anti-*Candida* factor produced by *Enterococcus faecalis.* BMC Microbiology 12-132. DOI: 10.1186/1471-2180-12-132.

2. **Raeesh M. Shekh,** P. Singh, S. M. Singh and Utpal Roy (2011) Antifungal activity of Arctic and Antarctic bacteria isolates. Polar Biology. 34:139–143. DOI 10.1007/s00300-010 0854-4.

3. **R. Shekh**, K.Upadhyay, S.M.Singh and U.Roy (2009) Inhibition of *Candida albicans* and Two Selected Gram-Negative Pathogens by Polar *Enterococcus faecalis* and *Carnobacterium* sp. Research Journal of Microbiology. 4(3):138-142.

Appendix- ii

Participation of Conferences/ Workshops/ Poster/ Oral Presentations

- Utpal Roy, Raeesh Shekh, Ramya R, Ram Lal and S M Singh "Antimycotic substances from wild-type *Enterococcus faecalis* APR 210 and *Bacillus subtilis* RLD 12.1" in the "Annual World Congress of Microbes-2" at Guangzhou China, 30th July-1st August, 2012. pp. 213.

- Raeesh M. Shekh and Utpal Roy "An Antimicrobial Protein from *Enterococcus faecalis"* poster presentation in "International Conference on Microbial Biotechnology for Sustainable Development" conducted by "Association of Microbiologists of India" at Panjab University, Chandigarh, November 3-6, 2011.pp 331-332.

- Raeesh Shekh and Utpal Roy "Inhibition of Multi-drug resistant *Candida albicans* by new extreme isolates" "National Conference on Emerging Trends in Life Sciences Research" attended on March 6th & 7th, 2009 at BITS, Pilani, pp 96-97.

- Raeesh M. Shekh "National Conference on Anaerobic Digestion and Renewable Energy through Microbes (ADREM 2009) attended on January 13th -15th, at Department of Biological Sciences BITS Pilani KK Birla Goa Campus Goa.

Appendix- iii

Brief Biography of Candidate

Personal Information

Name:	Mohd. Raeesh Shekh
Nationality:	Indian
Date and Place of Birth:	04/05/1978, Jaunpur, U.P.
Phone:	09373012102
Email:	*raeeshbiotech@gmail.com*

Education

M.Sc.	Dept of Biotechnology, Goa University	2007
B.Sc.	Kanpur University, Kanpur	2000
HSC	SIC M. Badshahpur, Jaunpur	1996
SSC	SIC M. Badshahpur, Jaunpur	1994

Work Experience

- Worked as SRF on a CSIR project entitled "Isolation and screening of microorganisms from extreme and unusual environments for new and novel antimicrobial peptides to combat some clinically important multidrug resistant fungal pathogens" since 16/03/2010 to March 2011.
- Worked on the same project as JRF since 15/03/2008 to 15/03/2010.
- Worked as JRF on a CSIR, project "Screening of marine microbes from the salterns of Goa for bioactive compounds "as JRF from 18th September 2007 to 14th march 2008

Academic Achievements

- Awarded CSIR SRF-Direct since April 2011 till November 2012.
- Qualified Graduate Aptitude Test (GATE) 2006, (AIR-583, SCORE-406)
- Selected in All India Combined Entrance for M. Sc (Biotechnology) in the year 2005.
- Got National Scholarship during Bachelor of Science

Appendix- iv

Brief Biography of Supervisor

Dr. Utpal Roy is the Head of the Department of Biological Sciences Group at BITS Pilani KK Birla Goa campus Goa, India. He is M.Sc. and Ph.D. (Microbiology) from National Dairy Research Institute, (Deemed University of ICAR), Karnal. He is involved in Teaching Courses like Genetic Engineering, Recombinant DNA Technology, Microbiology and Advanced Cell and Molecular Biology. In the last several years he has been involved in conducting Genetic Engineering / Recombinant DNA Technology related experiments for Undergraduate and Post Graduate Biotechnology Students. He has published and reviewed numerous Papers in the Journals of National and International Repute and also in the International and National Conferences / Symposiums. He is also Co-author of the Microbiology Review. His Major Research area includes Antimicrobials from Natural Resources.