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Computational Identification and Screening of Natural Compounds as Drug Targets against the Fish Pathogen, *Pseudomonas fluorescens*

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A B S T R A C T

Pseudomonas fluorescens is an important fish pathogen responsible for septicemia and ulcers in many fish species. Control of this pathogen in aquaculture systems is necessary to prevent the outbreak of disease in farmed fish. In this study, the complete proteomic information of different strains of *P. fluorescens* available in public domains was used for *in silico* subtractive analysis against zebrafish proteome to identify putative drug targets in *P. fluorescens*. The proteome set of *P. fluorescens* consisted of 32,664 proteins of which 7,794 were identified as non-paralogous protein sequences. A total of 163 essential proteins were identified in *P. fluorescens* with the majority of the proteins being involved in glycan biosynthesis and genetic information processing pathway. In the present study, two outer membrane proteins (OMPs), a surface antigen-D15 and a fimbrial biogenesis outer membrane usher protein, were identified as putative drug targets. A total of 2885 natural compounds available in ZINC database were virtually screened to identify their efficacy as putative drugs against the identified targets. Five natural compounds were identified, which bind to the putative drug targets and interfere with their functions. These compounds may be potentially useful as prophylactic and chemotherapeutic agents against *P. fluorescence* in aquaculture systems.

Introduction

The increasing global population is posing severe challenges to global food and nutritional security (Stentiford *et al.*, 2017). Aquaculture and fisheries play a leading role in nutritional and livelihood security, particularly in developing economies such as India. During the past three decades, global aquaculture production has expanded at an average annual rate of more than eight percent, from 5.2 million tons in 1981 to 62.7 million tons in 2011 and the contribution of cultured fish to total food fish supply grew from 9 percent in 1980 to 48 percent in 2011 (FAO, 2012). The estimated global production from aquaculture is expected to grow at an average annual growth rate of 4.5 percent over the period 2010–2030 (Brugère and Ridler, 2004). Presently, India is a major producer of fish through aquaculture and ranks second in the world with a production of 4.8 million metric tonnes (DAHDF, 2016). The increased aquaculture production is being

achieved through the adoption of new culture species, usage of supplementary feeds, intensive culture systems, etc., but it has also brought new challenges like increased incidence of known diseases coupled with the emergence of new pathogens causing economic losses to the sector. In aquaculture, the infectious diseases are considered to be major yield-limiting factor and industry-wide losses due to aquatic animal diseases exceed US$6 billion per annum (World Bank, 2014). This economic loss to the industry calls for an urgent need to develop prophylactic and curative measures. The Bangkok Declaration identified management of aquatic animal health as "an urgent requirement for sustaining growth" (FAO, 2000).

Among various bacterial pathogens of cultured fish, *Pseudomonas fluorescens* is of specific concern. It infects a wide range of fish species such as Indian major carps, common carp, eels, flounders etc and causes septicemia and ulcerative conditions (Swain *et al.,* 2007; Wang *et al.,* 2009; Khalil *et al.,* 2010). However, very little is known about the virulence factors or the pathogenicity of *P. fluorescens* to devise effective control strategies (Dagorn *et al.,* 2013; Zhang *et al.,* 2014). Currently, no commercial vaccines are available against *P. fluorescens*. Application of antimicrobials is the main strategy adopted to control this pathogen (Wang *et al.,* 2009). The use of antibiotics in fish culture systems invariably lead to the development of resistance by the bacterial pathogens. Various studies have reported isolation of the *P. fluorescens* resistant to oxytetracycline, tetracycline, amikacin and chloramphenicol (Sakai *et al.,* 1989; Markovic *et al.,* 1996; Darak and Barde, 2015). The increased incidences of antimicrobial resistance have prompted many researchers to study natural and natural like biologically active ligand compounds (Essawi and Srour, 2000; Haniffa and Kavitha, 2012).

The traditional method of drug discovery that follows disease based approach is expensive, and time consuming with significant time and research devoted to identifying potential ligands (Paul *et al.,* 2010). Computational methods help accelerate drug discovery by reducing the time consumed in the process by eliminating compounds that have limited chance of success (Wooller *et al.,* 2017). One such computational method makes use of differences in the proteins of the host and the pathogen for discovering potential drug candidates. The selected protein targets must be essential for viability or survival of the pathogen but should not have any well-conserved homolog in the host. Availability of complete proteome of many strains of *P. fluorescens* along with sufficient protein information of a well characterized zebrafish model presents an opportunity for computational analysis. This type of procedure has been successfully used against pathogens like *Mycobacterium tuberculosis* (Hosen *et al.,* 2006), *Klebsiella pneumoniae* (Georrge and Umrania, 2011), *Mycobacterium ulcerans* (Butt *et al.,* 2012), *Flavobacterium columnare* (Murali *et al.,* 2013), *Salmonella enteric* (Hossain *et al.,* 2017) and many others. The present study makes use of *in silico* subtractive approach based on the criteria of essentiality and selectivity to discover potential drug targets in *P. fluorescens*, followed by *in silico* virtual screening of drug targets against natural ligands to discover potential drug candidates.

Materials and Methods

Data collection

The complete proteome sets of *P. fluorescens* (6 strains) and the host, zebrafish (*Danio rerio*) were retrieved from the National Centre for Biotechnology Information (NCBI) (ftp://ftp.ncbi.nlm.nih.gov/genome) in FASTA format (Table 1). 2885 natural and natural like ligand molecules for *in silico*

virtual screening was downloaded from the ZINC15 database in SDF format.

Identification of essential genes

The complete proteomes of *P. fluorescens* and zebrafish (*D. rerio*) in FASTA format were subjected to CD-HIT analysis (http://weizhong-lab.ucsd.edu/cdhit_suite/cgi-bin/index.cgi) at 0.6 sequence identity cut-off to identify and remove paralogous sequences with more than 60% identity. The remaining sets of proteins were subjected to local BlastP program analysis (Altschul *et al.,* 1990) against zebrafish protein sequences with the expectation value (E-value) cut-off of 10^{-4}.

Proteins which did not show significant hits signifying non-homologous sequences were collected and blasted against the Database of Essential Genes (DEG) (http://tubic.tju.edu.cn/deg/) with an E-value cut-off score of 10^{-100}. From the blast results, proteins with amino acid length less than 100 were removed thus providing a dataset comprising of essential genes.

Functional analysis

Metabolic pathway analysis of the essential proteins was performed through single-directional-best hit method using KAAS server (http://www.genome.jp/tools/kaas/) of Kyoto Encyclopedia of Genes and Genomes (KEGG) to identify possible drug targets involved in specific metabolic pathways. Gene ontology analysis was carried out using Blast2Go Pro software.

Sub-cellular localization and topology prediction

Protein subcellular localization prediction involving the computational prediction of the location of protein residing in the cell was carried out using PSORTb V.3.0 program (http://www.psort.org/psortb/index.html) and the predicted membrane proteins were further analyzed in BOMP server (http://services.cbu.uib.no/tools/bomp/) to reconfirm that the identified probable drug target candidates were indeed membrane proteins. PRED-TMBB server (http://biophysics.biol.uoa.gr/PRED-TMBB/) was used to predict the topology and generate two-dimensional (2D) structural representation of Outer Membrane Proteins (OMPs).

Protein structure prediction of candidate targets

Phyre2 server employing homology modeling was used to predict the three-dimensional (3D) protein structure of candidate OMP targets. High resolution crystal structures of template proteins were searched using BlastP program from Protein Data Bank (PDB) based on homology (>30% sequence identity). The models were evaluated using Ramachandran Plot and Verify 3D programs. Protein structure representations were generated using Discovery Studio Client 4.1.

In silico virtual screening studies

In silico ADME (Adsorption, Distribution, Metabolism and Excretion) and toxicity screening were performed against natural ligand compounds. The binding site for interaction of the ligand with OMP receptors was created by selecting receptor cavities from receptor sites using define site module of Discovery Studio 4.1. Then, ligand compounds were prepared and minimized with maximum 225 numbers of conformations per ligand.

LibDocK module of Discovery Studio 4.1 was used for virtual screening. CHARMM force field was applied and energy minimized by steepest descent minimization for 1000 steps. The best-docked complex was selected based on scores.

Results and Discussion

The proteome set of *P. fluorescens* retrieved from the NCBI database consisted of 32,664 proteins comprising of paralogous and non-paralogous sequences. Paralogous sequences are duplicates created by gene duplication events within the genome resulting in similar functions. Since the function of an essential gene product is vital for the individual's survival, it is expected that essential proteins will have nil or a fewer number of paralogous (Giaever *et al.,* 2002; Gustafson *et al.,* 2006). Therefore, paralogous gene products can be removed or discarded while searching for essential genes. Through CD-HIT program, 27,870 proteins were excluded as paralogous sequences resulting in a dataset of 7,794 non-paralogous protein sequences which were used for further analysis. Identification of non-homologous pathogen proteins absent in host is the first major step in selecting the natural ligand compounds. This ensures that the putative drug compounds will not interfere with the host proteins and may not be toxic to the host. Local BlastP analysis against zebrafish proteome revealed that 4,116 out of 7,794 proteins were non-homologous with reference to the zebrafish proteome and were further selected for identification of essential genes. It is hypothesized that essential proteins are required for bacterial growth and survival and thereby, identification of essential proteins becomes important as the information can lead to potential drug or vaccine candidates (Lehoux *et al.,* 2001). In the present study, of the 4,116 non-homologous proteins, 163 proteins were identified as essential to *P. fluorescens* through DEG analysis. Proteins with less than 100 amino acids are usually not considered to be essential and are discarded from the analysis (Sakharkar *et al.,* 2004; Butt *et al.,* 2012). In the present study also, only those proteins which had an amino acid length of 100 or more were considered as essential proteins and used for further analysis. The average amino acid length in the identified essential proteins was 508 and the minimum similarity 45% or more with genes in DEG database. Similar results were observed in other studies *viz.,* Sakharkar *et al.,* (2004) reported 306 essential genes in *Pseudomonas aeruginosa*, Chong *et al.,* (2006) identified 312 in *Burkholderia pseudomallei*, Amineni *et al.,* (2010) identified 576 in *Leptospira interrogans*, Butt *et al.,* (2012) identified 424 in *Mycobacterium ulcerans*, Murali *et al.,* (2013) identified 238 in *Flavobacterium columnare* and 32 proteins in *Fusobacterium nucleatum* were identified by Habib *et al.,* (2016). The number of essential proteins or gene products identified in the present study was also found similar to those determined through experimental techniques in *Escherichia coli* (150 essential genes) (Jordan *et al.,* 2002) and *Bacillus subtilis* (203 essential genes) (Peters *et al.,* 2016) etc.

The essential proteins can further be classified into various functional categories based on gene ontology. In the present study the essential proteins of *P. fluorescens* were grouped into three categories *viz.,* those involved in molecular function, biological process and cellular component. The gene ontology analysis revealed that biological process and cellular component were found to be more dominant groups. Further the organic substance metabolic process and cellular metabolic process dominated the biological process group, while intracellular part, intracellular and membrane bound organelles dominated the cellular component group. Among the molecular function category, the majority were involved in protein binding and transferase activity (Fig. 1). In accordance with KEGG database, 154 out of 163 identified essential proteins could be mapped to metabolic pathways. Detailed analysis of metabolic pathways revealed that out of the 154 metabolic pathways, 13 metabolic pathways comprising of 24 proteins were found to be unique to *P. fluorescens* in

comparison to zebrafish (Annexure 1). Metabolic pathway analyses revealed that 43 proteins are involved in glycan biosynthesis, 30 in genetic information processing, 18 in carbohydrate metabolism, 12 in energy metabolism, 8 in bacterial secretion system, seven each in amino acid metabolism, signal transduction two-component system and cellular processes and signaling and 2 in lipid metabolism. The proteins involved in metabolic pathways can be targeted for identification of new drugs to control the pathogen. For example, bacterial secretion systems are used by bacteria to deliver virulence factors to host cells and hence are essential for its survival making it an important target for antimicrobial drugs (Chong *et al.,* 2006; Baron and Coombes, 2007). The present study identified eight proteins involved in the secretion system of *P. fluorescens*. Another important group of drug targets is enzymes involved in lipid biosynthesis. Inhibition of lipid biosynthesis can kill most gram-negative bacteria (Letain and Postle, 2007). The present study reports two enzymes, acetyl-CoA protein (YP_346849.1) and diacylglycerol-serine O-phosphatidyl transferase protein (YP_348607.1) involved in lipid biosynthesis pathway that can form potential drug targets and may be studied further.

One of the important groups of proteins identified as potential drug targets in bacteria are bacterial Outer Membrane Proteins (OMPs), the integral membrane proteins acting as the first line of defense in pathogens, characterized by an even number of β-strands ranging between 8 to 24 numbers arranged in an antiparallel fashion (Berg *et al.,* 2004; Walther *et al.,* 2009; Fairman *et al.,* 2011). OMPs undertake many different roles like acting as adhesion factors in virulence, channels for nutrient uptake, etc. (Tan *et al.,* 2008; Kim *et al.,* 2012; Rollaeur *et al.,* 2015).

Table.1 Proteome sets of the pathogen (*P. fluorescens*) and the host (zebrafish)

Sl. No	Particulars	Proteins
1	*P. fluorescens* (strain Pfo_1) (Silby *et al.,* 2009)	4122
2	*P. fluorescens* (strain Pf5) (Paulsen *et al.,* 2006)	4140
3	*P. fluorescens* (strain CHAO) (Silby *et al.,* 2009)	4434
4	*P. fluorescens* (strain SBW25) (Silby *et al.,* 2009)	5722
5	*P. fluorescens* (strain A506) (Loper *et al.,* 2012)	3768
6	*P. fluorescens* (strain F113) (Redondo-Neito *et al.,* 2012)	6115
7	*Danio rerio* (Zebrafish)	87,782

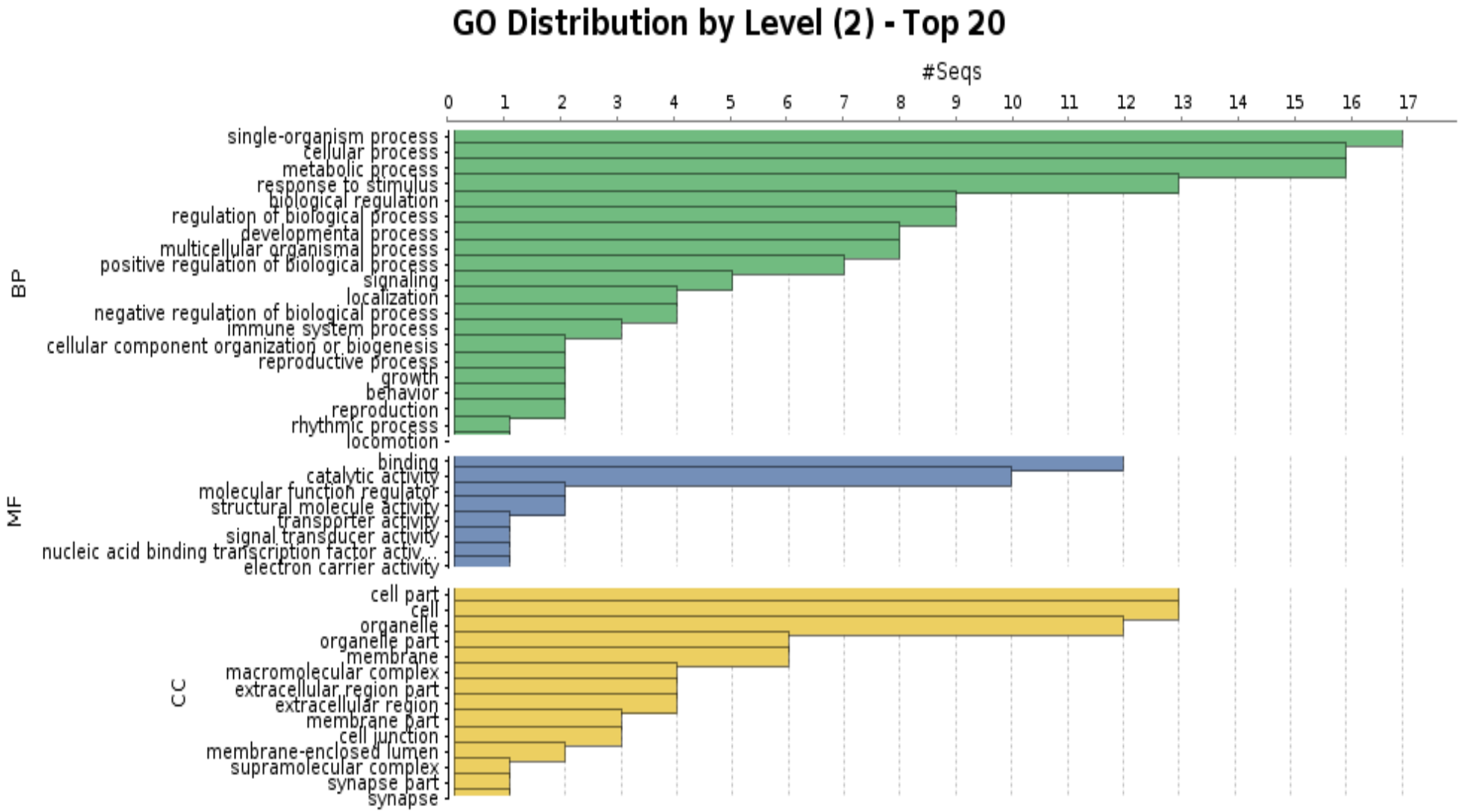
Table.2 Phyre2 result of homology modeling

Sl. No	Protein Name	Accession No.	Template	Similarity (%)
1	Surface antigen-D15	YP_346841.1	5D00	39.51
2	Outer membrane usher protein	YP_348773.1	3OHN	33.93

Table.3 *In silico* virtual screening result of selected ligands against two target OMPs

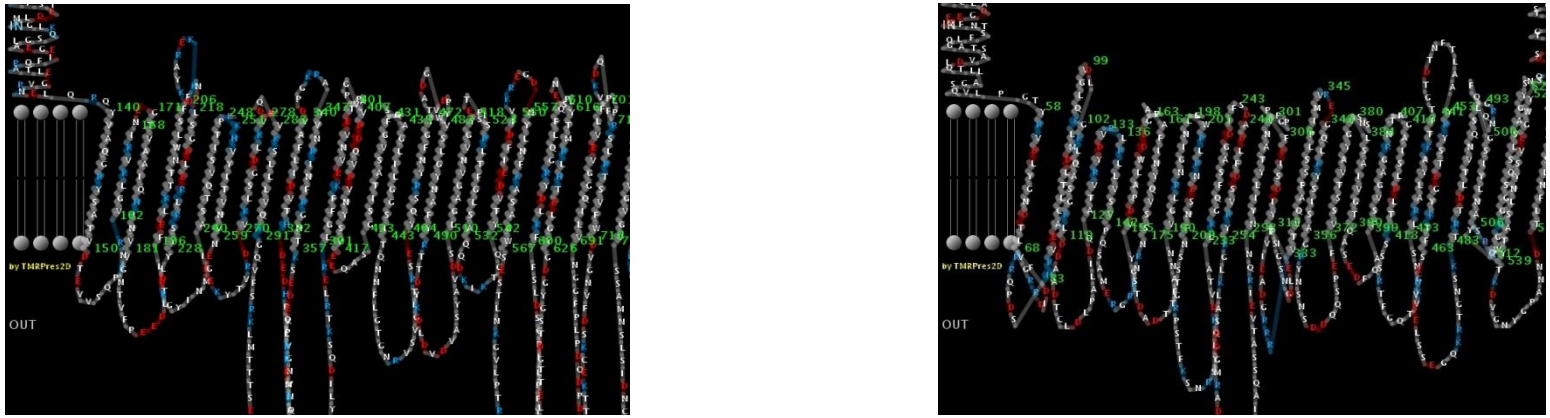
Sl.No	Zinc Accession Number	Ligand Name	Molecular Formula	LibDocK Score	Molecular Weight (g/mol)	Source
Surface antigen-D151 Protein (YP_346841.1)						
1	ZINC05179146	Columbianadin	$C_{19}H_{20}O_5$	84.899	328.3	*Radix Angelicae pubescenti* (Du huo herb)
2	ZINC05966742	Dihydrothebaine	$C_{19}H_{23}NO_3$	46.15	314.405	Biochemical
Outer membrane usher protein (YP_348773.1)						
1	ZINC00338132	Methoxyaporphin	$C_{18}H_{20}BrNO_2$	58.82	362.27	*Nelumbo nucifera* (Indian lotus)
2	ZINC05179146	Columbianadin	$C_{19}H_{20}O_5$	51.66	328.3	*Radix Angelicae pubescenti* (Du huo herb)
3	ZINC05762051	Ceylantin	$C_{16}H_{16}O_5$	43.78	288.89	*Atlantia ceylanica* (Sweet orange plant)

Fig.1 GO functional classification of *P. fluorescens*



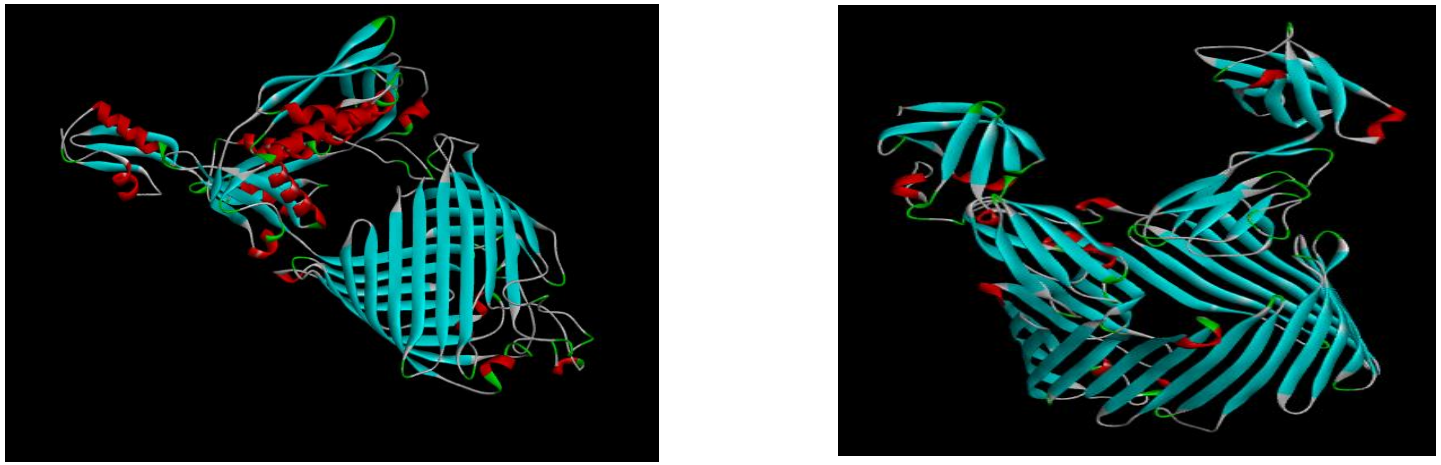
Histograms of frequency of sequences annotated to specific GO categories, biological process (BP), molecular function (MF) and cellular component (CC) are depicted by green, blue and yellow bars respectively

Fig.2 Predicted 2D structure of outer membrane protein (OMP) surface antigen (YP_346841.1) (A) and the fimbrial biogenesis outer membrane usher protein YP_348773.1 (B) of *P. fluorescens*



A B

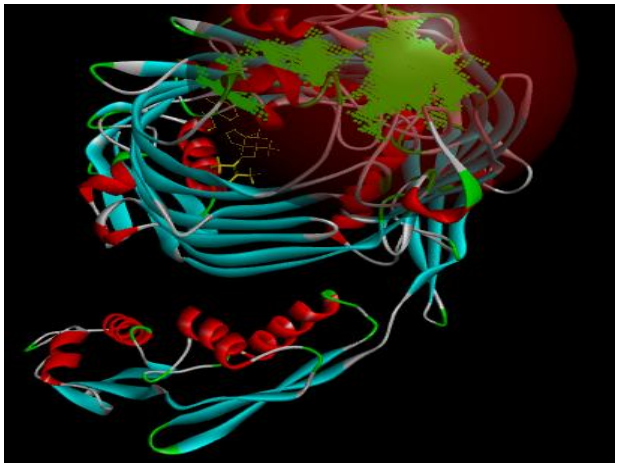
Fig.3 Homology models of OMPs of *P. fluorescens*: (A) Surface antigen protein (B) Outer membrane protein precursor



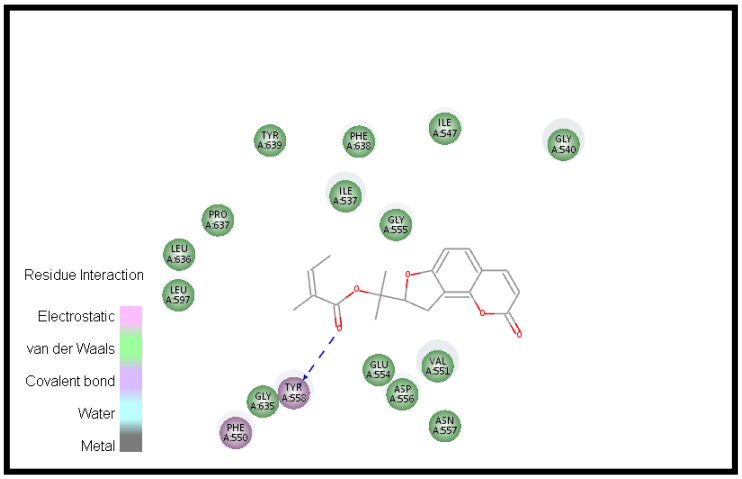
A **B**

Red colour – alpha helix Blue colour – beta strands Green colour – loops White colour - coils

Fig.4 Docking of ligand ZINC05179146 with surface antigen protein



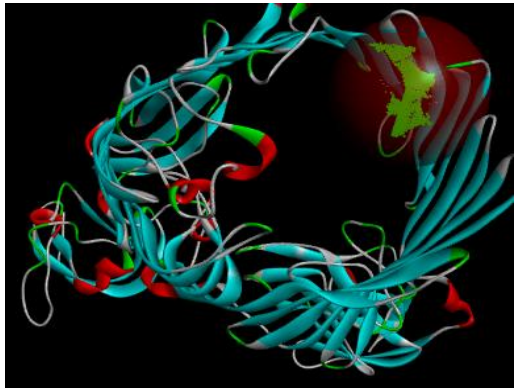
(A)



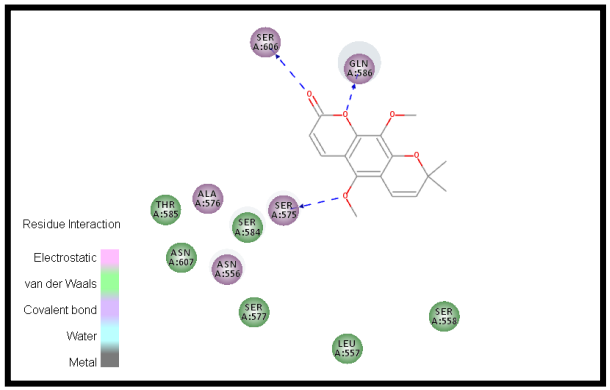
(B)

(A) 3D representation of docked ligand (Yellow colour-Docked ligand, Red sphere-docking site area)
(B) 2D representation of Non-bonded ligand interactions

Fig.5 Docking of ligand ZINC00338132 with outer membrane protein precursor



(A)



(B)

(A) 3D representation of docked ligand (Yellow colour-Docked ligand, Red sphere-docking site area)
(B) 2D representation of Non-bonded ligand interactions

Annexure.1 Proteins of *P. fluorescens* involved in unique metabolic pathways

Sl. No	Protein Accession Number	Protein Name	Pathway Name
1	YP_346200.1	rfaG;	Lipopolysaccharide biosynthesis
2	YP_346223.1	waaA;	Lipopolysaccharide biosynthesis and glycan biosynthesis
3	YP_346313.1	urease	Atrazine degradation and amino acid metabolism
4	YP_346601.1	UDP-N-acetylglucosamine	Peptidoglycan biosynthesis
5	YP_346845.1	UDP-N-acetylglucosamine	Lipopolysaccharide biosynthesis and antimicrobial peptide resistance
6	YP_346846.1	lipid-A-disaccharide	Lipopolysaccharide biosynthesis and glycan biosynthesis
7	YP_346852.1	2-dehydro-3-deoxyphosphooctonate	Lipopolysaccharide biosynthesis
8	YP_349370.1	UDP-2,3-diacylglucosamine	Lipopolysaccharide biosynthesis
9	YP_349899.1	UDP-N-acetylmuramate	Peptidoglycan biosynthesis
10	YP_349901.1	3-deoxy-manno-octulosonate	Lipopolysaccharide biosynthesis
11	YP_349903.1	tetraacyldisaccharide	Lipopolysaccharide biosynthesis
12	YP_350401.1	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide)	Peptidoglycan biosynthesis and prokaryotic defense system
13	YP_350403.1	UDP-N-acetylmuramoylalanine--D-glutamate	Peptidoglycan biosynthesis and selenocompound metabolism
14	YP_350404.1	phospho-N-acetylmuramoyl-pentapeptide-transferase	Peptidoglycan biosynthesis and vancomycin resistance
15	YP_350405.1	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine	Peptidoglycan biosynthesis and lysine biosynthesis
16	YP_350405.1	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine	Peptidoglycan biosynthesis, vancomycin resistance and lysine biosynthesis
17	YP_350407.1	Cell division protein	Peptidoglycan biosynthesis
18	YP_350520.1	penicillin-binding	Glycan and peptidoglycan biosynthesis
19	YP_350582.1	mviN;	Peptidoglycan biosynthesis and membrane protein
20	YP_350698.1	penicillin-binding	Peptidoglycan biosynthesis and drug resistance
21	YP_350698.1	penicillin-binding	Peptidoglycan biosynthesis and drug resistance
22	YP_350846.1	anthranilate	Phenazine biosynthesis and biofilm formation and quorum sensing
23	YP_346841.1	Surface antigen-D15	Membrane transport and OMP porin family
24	YP_348773.1	Outer membrane usher protein	Membrane transport, secretion system and cell motility

Computational means of identifying OMPs involve prediction of their location in the cell. In the present study sub-cellular localization prediction using PSORTb and BOMP led to the identification of two OMPs in *P. fluorescens viz.,* surface antigen-D15 (NCBI accession no. YP_346841.1) with 796 amino acids length and the second one was the fimbrial biogenesis outer membrane usher protein (NCBI accession no. YP_348773.1) consisting of 828 amino acids length (Fig. 2). The molecular weights of these OMPs were 87.7 KDa and 93.2 KDa for surface antigen-D15 and fimbrial biogenesis outer membrane usher protein, respectively. As tertiary crystal structures of these two OMPs were not available, homology modeling was used to build 3-D structures using a transferase protein and a membrane protein structure of *E. coli* as templates (Table 2 and Fig. 3). The predicted structures were validated by Ramachandran Plot and Verify Protein (Profile 3D) module of Discovery Studio software. The tertiary structures of the two OMPs were then used for virtual screening for potential drug targets. Rajalakshmi *et al.,* (2016) identified 8 OMPs in *P. fluorescens* and observed that their weight ranged between 3.7 KDa to 121.8 KDa. They further found them to be putative targets for developing vaccines to control septicemia.

From the ZINC15 database 2,885 natural ligands were selected and were used for ADME testing. These compounds were selected based on known toxicity and the ease of availability commercially. These 2885 natural ligands are available commercially from Specs Natural Products and Analyticon Discovery Natural Derivatives. ADME test evaluates the ability of a compound to be absorbed, distributed, metabolized and excreted from the body. Of the above mentioned natural ligands, 1,489 compounds successfully cleared ADME tests and were used for *in silico* virtual screening against the two identified OMPs, surface antigen-D15 (YP_346841.1) and fimbrial biogenesis outer membrane usher protein (YP_348773.1).

Docking is the computational simulation of how the targets OMPs bind to ligand compounds. Docking studies help to prepare a data set of ligands that successfully bind to the desired target. Each ligand can have more than one docked conformations called pose. In the present study the docking analysis was carried by employing a LibDocK program of Discovery Studio 4.1.

The docking analysis with LibDock identified a total of 1,546 poses during docking analysis of surface antigen-D15 protein with the set of natural ligands (Fig. 4A). Two compounds, Columbianadin (Chinese Du huo herb extract) and Dihydrothebaine (synthesized) had top LibDock scores and were identified as putative drug compounds. Non bonded interactions like hydrogen bonds, electrostatic force and Van der Walls interactions were studied with the help of receptor ligand interactions module of Discovery Studio Client 4.1 software.

Hydrogen bonds have been reported as facilitators of protein-ligand binding. Van der Walls force introduces a temporary change at the atomic level of interacting molecule resulting in temporary attractive force between interacting atoms. The Columbianadin (ZINC05179146) compound formed one hydrogen bond interaction with the Tyr^{558} residue. Residues Val^{551}, Gly^{555}, Ile^{537} and Gly^{540} were found to be mainly involved in Van der Walls interaction (Fig. 4B). Similarly, a total of 12,233 poses were identified in fimbrial biogenesis outer membrane usher protein of *P. fluorescens* using the same set of natural ligands (Fig. 5A). Based on the LibDock score, top three ligands, Methoxyaporphin (Indian lotus extract), Columbianadin (Chinese Du huo

herb extract) and Ceylantin (Sweet orange plant extract) were selected. Ceylantin (ZINC05762051) compound formed three hydrogen bond interactions with the residues, Ser^{575}, Ser^{606} and $Gln^{586.}$ Residues Ser^{584}, Thr^{585}, Asn^{607}, Ser^{577}, Leu^{557} and Ser^{558} were involved in Van der Walls interactions (Table 3, Fig. 5B).

The identified ligands were all characterized by low molecular weight compounds (less than 500 g/mol) with successful ADME tests. Of the five ligands that were selected, Dihydrothebaine is a biochemically synthesized compound while rest are natural extracts of plants like sweet orange, Indian lotus and Chinese du herb. One compound, Columbianadin could successfully dock into cavities of both the OMPs making it an important ligand. The small molecular weight, non-toxicity to the host, the ability to dock the target protein at lowest energy conformation, commercial availability and the most important being the natural compounds make the identified ligands in the present study promising drug compounds against *P. fluorescens*.

Health management in aquaculture has become very essential to enhance the production and profit. With the legal restrictions on the use of antibiotics and also the development of resistance by pathogens to the available antibiotics, there is an urgent need to identify new compounds to control the pathogens effectively. Five natural compounds identified in the present study are non-toxic to the host (fish) and have properties to control the *P. fluorescens* infection. Use of such natural antimicrobial products alone or in combination with lower doses of approved antibiotics or chemicals can help create sustainable aquaculture. Hence, the results obtained in the present study may further be validated by in-vivo studies and drug development purpose.

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