Screening of Indigenous Environmental Bacteria for Production of Bioactive Compounds (BaCs) and their Genes

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Abstract

Environment is a rich source of microorganisms, including bacteria and fungi that can produce significant bioactive compounds (BaCs). These microbes have been shaping their environment and thus the earth's biosphere for billions of years through an enormous range of BaCs. In Pakistan the knowledge regarding the indigenous microbes capable of producing BaCs is in very early stages. Our purpose of this study was to screen two microbes CMGN122 and CMGN370 isolated from Sindh, Pakistan for production of BaCs and their genes. Biological screening samples from microbial fermentation extracts were obtained after optimizing growth conditions and extraction procedures that capture BaCs produced. Column chromatography (CC) and TLC were used for isolation, compound identification and structure elucidation was done by different techniques of NMR and Mass Spectrometry. CMGN122 was identified as *Pseudomonas aeruginosa* (GenBank Accession No. JN969597), it was found to produce three different Phenazine compounds and pyrroloquinoline quinone (Pqq). CMGN370 identified as *Candida* sp. (GenBank Accession No. JN969598) showed production of two compounds Daidzein and N-(5-Acetylamino-pentyl)-acetamide. Genes for phenazines and PqqC were isolated and amplified.

During this study it was found that there are species which can be potential candidate for production of bioactive compounds.

Keywords: Phenazines, bioactive compounds (BaCs), *Pseudomonas*, *Candida*, Daidzein

Introduction

Microbes are the most numerous and ancient biotic communities which presence and metabolic activity is essential to the health and functioning of ecosystems and the whole environment through recycling of important elements, proper conditioning and fertility of the soil, pest and vector control and detoxification of naturally occurring and manmade pollutants (Overeas & Torsvik, 1998). These activities of microorganisms are based on their remarkable metabolic diversity and genetic adaptability. Our environment is a rich source of microorganisms including bacteria and fungi that can produce significant metabolites (primary and secondary). These microbes have been shaping their environment and thus the earth's biosphere for billions of years (Brock et al., 1994; Reith, 2002) through an enormous range of metabolic capabilities. These days as a result of far-reaching research, the study of microbial products is recognized as an essential component of natural products chemistry and as well as they are considerable resource for environment friendly compounds which have various industrial and agricultural applications. in Pakistan the knowledge regarding the indigenous microbes capable of producing microbial secondary metabolites is in very early stages. Our purpose of this study was to screen two isolates from soil and marine environment for production BACs.

Materials & Methods

Metabolite Production by CMGN122 and CMGN370
Growth Conditions CMGN122 and CMGN370 were grown M2 medium.

Pre-Screening
Entire culture was freeze-dried and the residue were extracted with ethyl acetate. TLCs were run in DCM:Methanol (90:10) from the crude extracts. Bands were observed.

Biological Screening
The crude extract was tested against *Bacillus subtilis*, *Staphylococcus aureus*, *Streptomyces viridochromogenes*, *Eschericia coli*, *Candida albicans*.

Brine Shrimp Micro-well Cytotoxicity Test
Cytotoxicity of crude extracts of CMGN122 and CMGN370 was checked with *Artemia salina*. The mortality rate M was calculated by using following formula;

$$M = \left[\frac{(A-B-N)}{(G-N)}\right].100$$

M= percentage of dead larvae after 24 hours
A= Number of dead larvae after 24 hours
B= Average number of dead larvae in blind samples after 24 hrs
N= Number of dead larvae before starting of the test
G= Total Number of Brine Shrimps

The extract, fractions or isolated compounds were considered inactive when the mortality rate was lower than 10%, from 10 to 59% as weakly active, from 60-95% as active and over 95% as strongly active.

Chemical Screening
Evaluation was done by separating the bands by number, intensity, UV reaction and color reactions with anisaldehyde / sulphuric acid spray on TLC.

Fermentation and Isolation of BACs
Three days old culture was harvested, mixed with Celite and separated into biomass and water phase via filter press by applying pressure. The biomass was extracted with ethyle acetate and acetone. The water phase was absorbed on an XAD-16 column and later extracted with methanol.

Different fractions obtained from CMGN122

Extracts were used and different compounds were isolated by fractionation on Silica Gel columns. Compounds were Identified by H^1NMR and C^{13}NMR. CMGN122 was found to produce phenazines. Genetic study was done for identified compounds of CMGN122.

isolation and Amplification of Phenazine Genes (phzE & phzF)
Primers used in this study were synthesized by Bio Basic inc. The degenerated primers for PCA gene phzE and phzF were sued for genetic study. ***fphzE*** (5'-gAA ggC gCC AAC TTC gTY ATC AA-3') and ***rphzE*** (5'-gCC YTC gAT gAA gTA CTC ggT gTg-3') as reported by Schneemann *et al.,* (2011) were used to amplify a highly conserved stretch of the *phz*E gene of approximately 450 bp. degenerated primers ***fphzF*** (5'-ATC TTC ACC CCg gTC AAC g-3') ***rphzF*** (5'-CCA TAg gCC ggT gAg AAC-3') were used to amplify approx. 427 bp. gene as reported by Mavrodi *et al.,* (2010).

Results

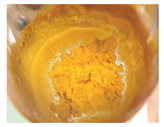
Pre-screening of CMGN122 and CMGN370 showed interesting bands on TLC which gave different colors with anisaldehyde spray and showed U.V absorbance.

Crude extract from CMGN122 and CCMGN370 were found to be biologically active. The crude extract showed activity against a number of microbes.

Biological Screening of Crude Extracts at 24 hr

		Zone of Inhibition in mm	
S. No	Test Organism	CMGN122	CMGN370
1	Bacillus subtilis	11	12
2	Staph. Aureus	21	17
3	Candida albicans	-	-
4	Eschericia coli	14	10
5	Chlorella vulgaris	-	11
6	Chlorella sorokiniana	-	11
7	Scenedesmus subspicatus	-	10.5
8	Streptomyces viridochromogens	-	-
9	Mucor miehei	-	12
10	A.coch	-	13
11	Rhizoctonia Solani	9	10
12	phytopathogenR2	43	-
13	phytopathogenY2	25	-
14	phytopathogenY3	20	-
15	phytopathogenY4	40	-
16	A.salina	100%	39.50%

CMC[illegible] 1-Hydroxy Phenazine, Phenazine Carboxilic Acid and Chlororaphine.

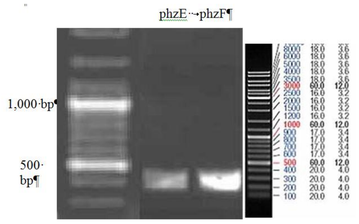


Hydroxy Phenazine

Phenazine Carboxilic Acid

Chlororaphine

Isolation and Amplification of Phenazine Genes
phzE and phzF were amplified and approx. 450 bp bands were obtained, which were sequenced.



PCR products of Phenazine genes phzE and phzF

Sequencing of Amplified PCR Product of Phenazine Gene
Sequencing revealed phzE (sequence submitted for Accession number) was 99% homologous with phenazine operon A-G of *Pseudomonas aeruginosa*, while phzF (Accession no. JX083238) was 92% homologous with phenazine operon A-G of *Pseudomonas aeruginosa*.

Discussion

The exploitation of microorganisms as a source for novel secondary metabolites production is still in its early life in Pakistan.

This study was carried out to screen microorganisms from different macro and microenvironments of Sindh for the production of BACs.

The failure of available antimicrobials to treat infectious diseases, sensitivity reactions and side effects of chemical derivative antimicrobial agents (Jamil, 2013) also persuaded investigators to study microorganisms to find out BACs to fight with infectious diseases. For the said purpose two isolates were selected i.e. CMGN122 and CMGN370 were chosen.

It was also observed that the isolates were producing more then one type of acid, including carboxylic acid, oxalic acid, and hydroxy acid. Aliphatic hydrogen acids test was also positive, same results were found by Cotter, (2003) and Khunajakr, (2008). During this present study TLC studies revealed that CMGN122 and CMGN370 produced indole-3 acetic acid and gluconic acids as well.

CMGN122 and CMGN370 were analyzed for compound profile by a number of chromatography techniques. CMGN122 was found to produce three bioactive phenazines 1-Hydroxy Phenazine, PCA and Chlororaphine. These results are in concordance with Leeflang *et al.,* (2002) and Kavitha *et al.,* (2005).

no plasmid was found in CMGN122 and CMGN370 which shows that the genes of inertest reside on genomic DNA. CMGN122 was further selected for genomic studies of phenazine gene operon (phzE anf phzF).

phzA, B and G are suppose to act in production of PCA but they cannot act with out phzF because PhzF product is thought to be the substrate of PhzA, B and G. (Parsons et al., 2004) Thus, for any study, PhzF would have to be present to initiate the reaction.

Amplified sequence of phzE and phzF genes showed 99% homology to complete genome and PhzA1-G1 and PhzA2-G2 gene cluster of *Pseudomonas*.

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