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ORIGINAL ARTICLE

Exploring Biodiversity for Prevention and Control of Cancer and other Non-Communicable Diseases using Indigenous Environmental Resources: An Interdisciplinary Research

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Abstract

Background: An interdisciplinary research of public health, biomedical and pharmaceutical sciences is needed for integrating qualitative and quantitative researches undertaken. It hence requires focus on public beneficence for non-communicable diseases. **Purpose:** To study anticancer activities of soil samples of Central India and its stability for applied public health use. **Material and Methods:** Screening on Actinomycetes isolates obtained from rural and urban farm soils illustrating arginase production was conducted from equated soil samples of geo-representative localities and adjoining areas of Bhopal, India. Enrichment Technique (CDSEA) was used for detection of extracellular production of L-arginase and their anticancer activities using MTT 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay along with characterization and probiotic properties of selected isolate. **Results:** L-Arginase activity quantified by ornithine (21.06-117.92 U/mg) was found in isolates BRD-21, KAR-73, BHA-162, BAR-199, ARH-210, HAB-228. Urea release (15.88 – 59.79 U/mg protein) depicted L- arginase activity in crude enzyme samples. It shows noticeable anticancer activity. Morphological and biochemical characterization of these isolates revealed metabolic diversity. Isolate KAR 73 produced collagenase (specific activity 57.8 U/mg), L-asparaginase (specific activity 116 U/mg) and L-arginase with tolerance to higher temperature (45°C) and salt concentration (2-8% w/v). Equal concentrations of crude L- arginase from these isolates inhibited growth and proliferation of colorectal adenocarcinoma cell lines (19.99%-38.65%) under *in-vitro* conditions. **Conclusion:** Arginine depletion through arginase activity is evidenced for potential effectiveness in cancer treatment especially adenocarcinomas and squamous cell carcinoma. It is useful for wider public health purposes.

Keywords

Actinomycetes, Anti-adenocarcinoma, Arginase, Ornithine, Probiotic, Urea, Metabolic diversity

Introduction

Multifaceted quality care is to be prioritized for health insured persons and beneficiaries of community centric health care services especially those from unreached, difficult and tribal areas. Abundance of actinomycetes in fertile soil, unfertile soil and sub-soil is dependent on characteristics of collection site, climatic conditions and diversity of locational microflora. Use of such actinomycetes for controlling non communicable diseases in cost effective manner needs to be explored for gains in resource constrained environment (1). This poses

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challenges of analysis for its use towards applied purposes (2). L-arginase activity has vital role in range of physiological conditions predominantly liver injury, acute neurological disorders, cell growth, regeneration, asthma, hepatitis infection, cell proliferation, cancers & rheumatoid arthritis. It has been endeavored to understand usage of actinomycetes for human beneficence in terms of reducing morbidity and mortality profile (3). The research for potentials of Actinomycetes continues as area of interest in technical laboratories (4). Easy and abundant availability of Actinomycetes is noticeable in all geo-locations and environmental conditions (5, 6).

Although anticancer enzyme viz., L-asparaginase is best obtainable from marine samples and slaughter house sample, the feasibility of such samples is still comparable with those from agriculture field sample (7). Hence, the samples collected from soil of urban and rural fields are equally good to assess their antifungal and anticancer properties (8, 9). Actinomycetes has wide metabolic potentials due to presence of plasmids. This study opens opportunities for further and extensive researches to address etiological factors leading to accelerated non communicable disease morbidities, the public health challenge of present times. This study aimed to assess arginase activity of Actinomycetes isolated from soils of Bhopal and adjoining area. The objective was to assess production of ornithine and urea for assessment of *in-vitro* activity against adenocarcinoma.

Material and Methods

66 soil samples were collected from 33 soil sample collection sites, as identified by convenient sampling method over five-kilometer radius in and around Bhopal city. These soil samples were used for isolation. Czapek's Dox Soil Extract Arginine agar medium, Yeast extract malt extract agar [ISP-2], ninhydrin reagent, MTT dye, Dulbecco's Modified Eagle's medium (DMEM), Man Rogosa Sharpe (MRS) broth, modified Bennett's medium. *In vitro* study, comparative study, enrichment technique, regionally representative samples from the study area were collected.

Isolation of actinomycetes was done using Waksman's serial dilution method. The samples were serially diluted in sterile distilled water up to 10^{-2} to 10^{-3} dilutions and 0.1 mL aliquots of each dilution were spread over the surface of Czapek's Dox Soil Extract Arginine agar medium (CDSEA). The plates were then incubated at 28±1°C for 7 days. The colonies typical of actinomycete growth i.e., those possessing chalky and powdery appearance, radiating growth, irregular and fuzzy edge, leathery texture etc. were isolated and purified by streaking. All the experiments were done in triplicate set. The isolates of Actinomycetes were maintained as working and stock cultures on Yeast Extract Malt Extract agar slants. All the isolated and purified Actinomycetes (equivalent to 2 x 10^3 spores/ml) were grown in CDSEA broth at 28±2°C for 8 days for detection of extracellular production of L-arginase. Quantification of extracellular L-arginase produced by Actinomycetes was done in cell free culture broth. Enzyme Units was calculated by assessment of factor obtained from ratio of Volume of reaction mixture (V_m) and Volume of enzyme (V_e) multiplied with inverse of time in hour (t). This factor is thereafter multiplied with amount of ornithine or urea released due to enzymatic action (T-C). Arginase activity was measured by the modified method of Roman and Ruy's, 1969. Reaction was carried out at 37°C for one h and stopped by adding ice cold Trichloro acetic acid TCA (10% w/v). Optical density was measured at 515 nm using Pico drop Spectrophotometer (PICOPET 01, UK) against the reagent blank. Estimation of urea was done by method given by Chen and co-workers with slight modification (10). The absorbance was read at 525 nm using Picodrop spectrophotometer. 1 unit of enzyme activity as produced 1µmole of urea in 1 hour at 37°C. The protein content was determined according to the method of Bradford using Bovine Serum Albumin as standard.

The cell line [HT-29 (human colorectal adenocarcinoma)], was procured from the National Centre for Cell Sciences, Pune and cultured and maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 10% (v/v) FBS at 37°C 5% CO_2. Arginosuccinate synthetase (ASS) deficiency in cell line was confirmed using RT-PCR. After trypsinization cell (99% cell viability) was used for cytotoxicity assay of L-Arginase using MTT assay. Dark-blue formazan crystals were dissolved in 1-propanol, and the absorbance was measured at 570 nm by ELISA method. Cultural, morphological, physiological and biochemical properties of the selected strains were studied according to methods of Shirling and Gottlieb, 1966. Pattern of growth, texture, colour of aerial mycelium, formation of concentric rings and production of diffusible pigments were predominantly recorded on yeast extract malt extract agar medium (ISP-2) for 14 days at 28 ± 1°C followed by colony and pigment identification by Rayner's colour chart, as has also been observed in the study of species showing colour variations (11).

Production of biomass of actinomycetes was done on CDSE broth medium. The biomass was then washed and hydrolysed using 6N HCl and boiled at 100°C overnight. The hydrolysate was filtered to remove debris and the extract was mixed with equal amount of water, thereafter dried in vacuum evaporator. Residue was re-dissolved deionized water and dried. Sample (3 µL) was loaded on cellulose coated TLC sheets and eluted. The identification of Actinomycetes is enabled through cost effective and rapid indigenous screening methods and is therefore positive influencer for commercial gains (12). Actinomycetes possessing potential to produce L-arginase were further characterized for bile tolerance, acid tolerance, salt tolerance, and phenol tolerance. Enumeration of viable cells was done by appropriately diluting (10-fold) harvested broth. Growth was also monitored by measuring absorbance with spectrophotometer. Log phase culture of Actinomycetes was inoculated in yeast extract malt extract agar. The growth was graded positive (+), weak positive (W+) and negative (-) by comparing growth in control slant. Cross streak method was used to test the antimicrobial activity of actinomycetes against test fungi *Microsporum gypseum* (MTCC 6041), *Fusarium oxysporum* (MTCC 1755). The plates were observed for inhibition in growth of fungi after 5 days at 28° ± 2°.

Statistical analysis was done using Epi Info™ Software. The analysis was done for experimental and control groups. Graphical representations of the data were performed using GraphPad Prism.

Results

All tested soil sample exhibited presence of Actinomycetes and 231 Actinomycetes were isolated and purified on self-designed medium CDSEA (*Figure 1*). The arginase activity was observed in 166 isolates. Arginase activity of potential Actinomycetes was measured as urea production and ornithine production. The arginase activity was found >20U/ml in 22 isolates among which isolate numbers 21, 73, 162, 199, 210 and 228 displayed activity more >50 U/ml and are hence graded as potential arginase producers.

The typical growth pattern of KAR-73 is identified through gray coloured spore mass, positive rectiflex and cell wall L-DAP. The viability of KAR-73 is illustrated by its utilization of most of the common carbon sources viz., lactose, fructose, sorbitol, salicin, sodium gluconate and dextrin in addition to acid viz., methyl pyruvate, D-Sacchric acid, D- gluconic acid and quinic acid (*Table 1*).

The cell cytotoxicity of crude arginase against HT-29 ranged between 19.99% to 38.65% for selected isolate no. 21, 73, 162, 199, 210, 228 with its highest level observed in isolate KAR-73 considering crude arginase to be 50U/ ml. Maximal specific activity for ornithine and urea is observed with KAR-73 as 117.92 U/mg and 59.79 U/mg as compared to other short listed potential Actinomycetes.

The Probiotic potentials of *Streptomyces plicatus* KAR 73, as identified here, is within the above said status, while showing high salt tolerance (87±2.3% growth for

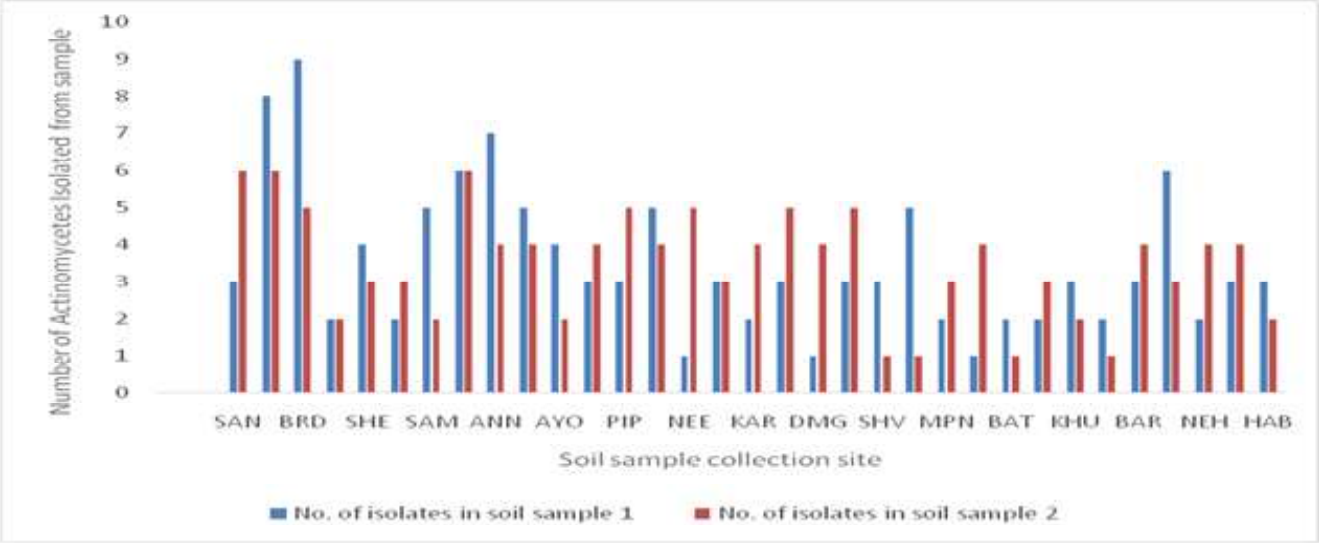

Figure 1: Isolation of Actinomycetes from Different Soil Samples on Czapek-Dox Soil Extract Arginine Agar Medium Incubated at 28ÚC for 3-7 Days (Sites Coded for the Research Conducted viz. SAN, BRD, SAM etc)

Table 1: Characterization of Potential L-Arginase Positive Actinomycetes Isolates BRD-21, KAR-73 and BHA-162

S. No.	Test*	BRD-21	KAR-73	BHA-162	S. No.	Test*	BRD-21	KAR-73	BHA-162
1	Spore chain [Rectiflex (R)/ Spirals (S)]	(S)	(R)	(S)	20	L- Malic acid, D- Saccharic acid, Methyl pyruvate	+	+	+
2	Color of Spore mass	Gray	Gray	Gray	21	L-Lactic acid	+	-	W+
3	Melanin production on tyrosine agar	w+	+	w+	22	D- Malic acid, α-keto glutaric acid*	-	-	-
4	Indole production, Methyl Red test, VP, Citrate utilization, H_2S production, Lecithinase Nitrate reduction*	-	-	-	23	D- Glucuronic Acid, L-Galactonic acid Lactone, D-Galacturonic acid*	D	+	+
5	Hydrolysis of starch, gelatine, Gelatine liquefaction, casein, Lipolysis, Catalase test	+	+	+	24	Mucic acid, Glucuronamide*	D	-	D
6	Growth in NaCl (%)	2-12	2-11	2-12	25	L-Lactic acid methyl ester	W+	-	-
7	Growth at pH	5-11	4-11	6-11	26	Pyroglutamic acid	-	+	+
8	Growth temperature (°C)	15-42	15-47	15-42	27	Quinic acid, D-Gluconic acid	D	+	D
9	Growth in presence of inhibitors and Antibiotic				28	Xanthine degradation	-	++	++
10	Sodium Azide, 0.01%, 0.1% *, Neomycin (50 µg/ml), Troleandomycin, Hippurate utilization*	-	-	-	29	Utilization of carbon source Adonitol, Sodium gluconate, Insulin, Sorbitol, Salicin, D-Cellebiose, Dulcitol, Mannitol, Erythritol, Arabitol	-	+	+
11	0.1% Phenol, Lithium chloride	-	+	+	30	D- Arabinose, Xylitol Utilization	+	-	+
12	0.001% Potassium tellurite, Fusidic Acid	+	-	+	31	D-Melibiose, Fructose, D-Maltose, Galactose, Inosine	+	+	-
13	0.001% Thallic acid, Rifampicin (50 µg/ml), Penicillin G (10IU) *, Olendomycin (100 µg/ml), Sodium lactate (1%)	+	+	+	32	L-Arabinose, Lactose, Mannose, Sucrose, Gentiobiose, D-Trehalose, Melezitose, meso – Inositol, Xylose, Glucose, Glycerol, α Methyl-D-Mannoside, Sucrose, Rhamnose	+	+	+
14	0.01% Thallic acid, Nalidixic Acid	-	+	-	33	L-Fucose. D-Fucose, 3-Methyl-D-Glucose	D	-	D
15	Lincomycin	D	-	W+	34	Raffinose	++	-	-
16	Vancomycin, L-Serine	D	-	-	35	Dextrin	-	++	++
17	Utilization of nitrogen source L-Tyrosine, L-lysine, L-Histidine*, L-glutamic acid	++	++	++	36	Malonate, ONPG, Esculin	-	-	-
18	L-arginine, L-Hydroxyproline, Elastin degradation, Sorbose, Arbutin Degradation, L-Valine	+	+	+	37	L-arginine, L-Hydroxyproline, Elastin degradation, Sorbose, Arbutin Degradation, L-Valine	+	+	+
19	L-Aspartic acid	-	+	+	38	Cell wall L/ D -DAP	L	L	L

*All the experiments were performed in triplicate set

Table 2: Probiotic Potential of Streptomyces plicatus KAR-73

Probiotic Properties	Growth (Grading)#	Growth (%)
Bile tolerance		
0.3%	+	69.14±3%
0.5%-0.8%	-	0
Acid tolerance		
2 pH - 4 pH	w+	54.32±5%
5 pH - 7 pH	+	98.1±1%
Salt tolerance		
2-8 %	+	87±2.3%
8-11%	w+	44.9±3.7%
12-14%	-	0
Phenol tolerance		
0.1%	+	82.89±4.2%

w+: Weak Positive; +: Positive; - : Negative

2-8% w/v), phenol tolerance (82.89±4.2% growth for 0.1%), bile tolerance (69.14±3% growth for 0.3 %) and acid tolerance (98.1±1% for 5-7 pH) (*Table 2*).

In addition, the range of tolerance for acid (2-7pH) and salt tolerance (2-11%) is also high. The physical characteristics of colony and/ or their count is not found to be in resonance with their individual genetic makeup and capacity to produce metabolic enzymes. The enzymatic activity is observed through the evidenced utilization of all nitrogen sources except L-Serine and L-Cystine. The antifungal properties of actinomycete KAR-73 is clearly shown by rapid progressive and typical growth inhibition (*Microsporum gypseum*: 8 mm inhibition in growth; *Fusarium oxysporum*: 10 mm inhibition in growth) due to production of secondary metabolites on SDA media plates.

Discussion

With ever expanding and challenging horizons of cancerous risk factors evidenced through life style change, urbanization, migration, unaddressed socio-economic issues and the complex amalgamation of multi-factorial causation induced disease phenomenon, there is intervention requirement for catering to the ever-increasing burden of gastro intestinal cancers (13).

The comprehensive approach to analysis and interpretation of herein obtained results has enriched this study on multifaceted aspects generally deficient in any singular study conducted elsewhere and reviewed here. While, sample collection was done from scattered geological areas spread over more than 30 kilometers, it is interesting to note that none of the random samples had zero count of Actinomycetes. It is also noteworthy that the density of isolate had not any association with the location for the type and quality of results, when inter-isolate comparison was studied. The possibility of getting new strains of Actinomycetes is still unexplored from difficult to approach areas, biodiversity hotspots and restricted areas, thereby provides abundant opportunity for identification of novel strains with neo applied purposes (14).

As evidenced here, the L-arginase positive Actinomycetes comparably studied for three short listed strains namely BRD-21, KAR-73 and BHA-162 are indicative of reasonably efficiency enriched types of Actinomycetes. Although KAR-73 like BRD-21 and BHA- 162 is not found positive for nitrate reduction, it is surely able, however, to hydrolyse starch gelatine and casein and also is able to liquefy gelatine and lipids. The area specific isolate clusters have also not yet been studied for their cumulative benefits and are therefore rich resources requiring new strategic protocol-based researches. The impressive growth of KAR-73 in presence of Rifampicin, Oleandomycin, Penicillin G, Nalidixic acid, Sodium lactate and lithium chloride illustrates the usefulness of Actinomycetes for even those under tertiary or terminal care *(Table 1)*. Recognising use of Actinomycetes, it is imperative to extensively study the potential, distribution and commercial applicability of arginase derived from Actinomycetes from their natural habitats (15).

The essentiality, desirability and effectiveness of probiotic potential is dependent on the grade and percentage of Actinomycetes growth predominantly for tolerance of bile, acid, salt and phenol. Its noteworthy that the spectrum of Probiotic potential of *S. plicatus* KAR 73 is found to be positive for one or more strata specific properties with weak positive growth for acid and salt tolerance as well and hence is suited on the parameters of fitness for survival and growth of Actinomycetes. Having conducted *in vitro* or *in vivo* experiments for usefulness of Actinomycetes should necessarily follow the randomized double-blind placebo-controlled trials for organ, system and region-specific anticancer activities with assessment of magnitude and completeness of resolution gained by using the developed Probiotic (16).

Strength of Actinomycete KAR 73 showing its equally appreciable lytic potential for slow and fast-growing pathogenic fungi. The characteristic of culture from

isolated and short listed Actinomycetes has usually been replicated during various studies using different protocols, although for repetitive outcome measures. The proven antimicrobial effect of isolates from known enriched reservoirs of actinobacteria has although been evidenced, its anticancer activity even from those isolates from collection sites of Urban residential areas is an encouraging marker of bio-abundance (17). This research is hence unique because of the directionality, quantum and its applied purpose.

The MTT assay has clearly demonstrated the anticancer potential of L-arginase from different Actinomycetes, although extent of growth inhibition differed among isolates. Resembling our *in vitro* study, a UK based study conducted *in vivo* and *denovo* conditions confirms that arginine deprivation is the key for targeted oncotherapy (18). The efficiency, effectiveness and bio-availability of ADI enzyme is more important for being identified as possessing anti-tumour properties (19). The present study has proven to be of highly antitumor potential as evidenced through high inhibition level (38.65%) in growth of HT-29 cancer cell lines under *in vitro* conditions.

Although this study has emphasized mainly on usefulness of arginase for anticancer activities, the arginase has also been identified to have important role in hypertension, diabetes vascular disease, atherosclerosis, myocardial-ischemia-reperfusion injury, aging & cellular senescence, erectile dysfunction, ischemic stroke, Alzheimer's disease, and traumatic brain injury. However, the challenge of arginine inhibition induced abnormalities in human physiology and pathology remains to be addressed (20). In addition, the wider applicability in resource constrained settings of India shall save the additional burden of out of pocket expenses of the deprived population. Hence, the study results, being in resonance with similar other studies, are indicative of extensive use of bio-diversified resources and their optimal cost-effective use.

Conclusion

In conclusion, herein identified and short listed actinomycetes isolate is promising not only for its anti-carcinogenic activities, but its commercial utility even vide singular use of ornithine production is directed for public health gains. Having noted its tolerance for bile salts, although selective, it provides a ground for exploring its usage in hepatic carcinomas and injuries. The benefits of actinomycetes are hence inferred to be of mass applicability due to their easy availability and better acceptability coupled with wide range of functionality for pH, temperature and sources of carbon, nitrogen and inorganic substances. This study paves way for intensification of research involving rural and tribal areas to gather potentially useful resources from their rich diversity of environmental treasure. The native enzyme exploration is thus of importance for public health applications in varied settings. The study, being interdisciplinary, opens vistas of opportunities for conduct, infer and apply scientific results from the easily available in-country resources towards public health applications for larger benefit of deprived population especially suffering from cancer and other non communicable diseases.

The study identifies Actinomycetes KAR-73 as potent anticancer agent and may need supportive and expectedly encouraging evidences for potential application in other non-communicable diseases like myocardial infarction, diabetes, ageing and Alzheimer's disease. Hence, the outcome of this study underlines the importance of herein identified Actinomycetes for cost effective commercial production of its products by stakeholders of health, agriculture and scientific research institutions.

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Conflicts of Interest

There are no conflicts of interest.

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