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ISOLATION OF ACTINOMYCETES FROM RHIZOSPHERE SOIL: A COMPLETE APPROACH

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ABSTRACT

Objectives: The current study focuses on the isolation of actinomycetes from various rhizosphere soil samples by using different pretreatments and selective medium.
Methods: A actinomycetes were isolated from rhizosphere soil samples of different plants by soil pretreatment, physical and chemical treatment and grown with and without an antibiotic medium.
Results: A total 69 of actinomycetes strains were isolated from rhizosphere soil samples by using physical and chemical pretreatment, including dry heat, centrifugation, phenol treatment, enrichment in a nutrient medium, calcium carbonate treatment, and selective nutrient medium with and without antibiotics. Physical treatment and chemical concentration effect the isolation of actinomycetes. It is found that dry heat at 40°C an effective pretreatment, centrifugation eliminates the unwanted soil debris, phenol and calcium carbonate are more effective chemical treatments, antibiotics medium is useful for isolation of actinomycetes by preventing bacterial and fungal growth. It is found that actinomycetes are rich in rhizosphere soil and have the ability of produce plant growth hormones, bio-nutrients, bioactive compounds that are useful to promote soil fertility and plant growth.

Keywords: Actinomycetes; antibiotics; calcium carbonate; rhizosphere; bioactive compounds; dry heat.

INTRODUCTION

Microorganisms constitute an exhaustible reservoir of compounds including plant growth hormones, bioactive agents, bio control agents, mineral solubilizing agents, carbohydrate degrading enzymes, chemical degrading enzymes, detoxifying compounds, acid and alkali neutralizing compounds, pharmaceutical, agricultural and industrial important chemicals. These compounds are produced by bacteria, fungi, and actinomycetes. Among the microorganisms actinomycetes are a prominent and major place to produce type of compounds. Actinomycetes are found in large numbers in soils, waters, lakes, river bottoms, manures, composts, sea shower and dust as well as on plant parts, residues and food products. The ability of actinomycetes to produce

different types of bioactive substances has been researched and utilized in a comprehensive manner in industrial and research laboratories [1-8].

Plant growth depends on many factors including soil fertility, nutrient and mineral availability in soil, and external factor including climatic conditions. Plants suffer the challenge to get the desired nutrient for growth and development due to immobility. The plant growth depends on the external supply of fertilizers and available nutrients in the soil. Fertile soil having rich of nutrients, minerals, plant growth trace metals, salts buffering comments and microorganisms etc. Microorganisms play an important role to enhance soil fertility by dissolving insoluble components into a soluble form, nitrogen fixation to make availability of nitrogen for plant growth, accumulate nutrient sources, produces growth-enhancing chemicals and acids etc. [1,4-8].

Actinomycetes are prominently available in the soil of different habitats and plants parts. Many research works were carried out on the isolation of actinomycetes from soil with different methods, very few and limited articles are available on isolation of actinomycetes on the plant parts. Researchers employed different methods of pretreatments, physical and chemical treatments to isolate actinomycetes from soil, and very limited from plant parts. Actinomycetes are rich source of agriculture, medicinal, pharmaceutical and chemical important chemicals, hence the immense need for isolation of actinomycetes from different habitat and screening for their ability to produce such important chemicals [9-10]. Considering the present scenario, the present work is mainly focused on isolation the actinomycetes from soil using different methods.

MATERIALS AND METHODS

Rhizosphere Soil Sampling and Processing

Soil samples collected from near the Roots of *Vitis vinifera L.* (Grape) and *Triticum aestivum L.* (Wheat) in a sterile plastic bag using a sterile spatula. The collected samples were air-dried in a laminar air flow unit for 24 hrs. Dried samples were preserved in 2 to 8°C until process.

Isolation of Actinomycetes

Different pre-treatments methods, physical and chemical methods employed for isolation of actinomycetes using selective nutrient medium. [4-9].

Medium for isolation of actinomycetes

Glycerol Asparagine Agar plates, Yeast Extracts Malt Extract Dextrose Agar plates were used and the same media with and without antibiotics [4-7].

Treatment for Rhizosphere soil samples

Dry heat

One gram of samples was heat dried at 30, 40, 50 and 60°C for 30 minutes. Each sample was mixed in the 9 ml sterile normal saline water and centrifuged at 4000 RPM for 4 minutes, and the supernatant were serial was serial diluted and plated on the various medium as described in earlier section.

Calcium carbonate treatment

In a separate test tube, 1 gram dried sample of soil as well as root was mixed with 0.5%, 1%, 2 % and 4% calcium carbonate and tubes were incubated at 30°C for 48 hrs. Treated soil and root samples were mixed in 9 ml normal saline solution stored at static for 3 hrs. 1 ml supernatant was serially diluted and plated on various media plates. Plates are incubated at 28°C for 10 days.

Combination of dry heat and Calcium carbonate treatment

One gram of soil sample samples heat dried at 30, 40, 50 and 60°C for 30 minutes and treated with 1% calcium carbonate and incubated for 30°C for 48 hrs. Treated sample serial diluted and plated on the various medium. Plates are incubated at 28°C for 10 days.

Phenol treatment

Each 1 gram air dried sample of soil and root weighted in a separate test tube and was mixed with 0.25%, 0.5%, 1.5% and 2% Phenol and tubes were incubated at 30°C for 2 hrs. Treated samples

were mixed in 9 ml normal saline was serially diluted and plated on various media plates. Plates are incubated at 28°C for 10 days.

Dry heat and Phenol treatment

In a spate test tube,1 gram of sample heat dried at 30, 40, 50 and 60°C for 30 minutes and treated with 1% Phenol and incubated for 30°C for 48 hrs. Treated sample serial diluted and plated on the various medium. Plates are incubated at 28°C for 10 days.

Enrichment treatment

In a spate test tube,1 gram of sample mixed with 9 ml starch casein broth with antibiotic and incubated for 30°C for 48 hrs. Treated sample serial diluted in normal saline and plated on the various medium. Plates are incubated at 28°C for 10 days.

Preliminary identification of actinomycetes

Muddy and dry colony considered as actinomycetes and it confirmed by observing filamentous hypal growth in microscopy [11-12].

Storage and preservation of actinomycetes

Isolated actinomycetes strains were subculture on Yeast Extract Malt extract slants, and slants incubated for 10 days at 30°C. Grown culture slants were kept in 2 to 8°C until further use.

RESULTS AND DISCUSSION

Isolation of Actinomycetes

Isolation results of dry heat treatment

A total of 16 actinomycetes strains were isolated from rhizosphere soil of Grape and wheat plant by dry heat treatment. Detailed isolation results shown in Table 1. It is noticed that the 40 to 50°C is dry heat treatment is effective for isolation of actinomycetes.

Isolation results of calcium carbonate treatment

A total of 22 actinomycetes strains were isolated from rhizosphere soil of Grape and wheat plant by various a concentrations of calcium carbonate of treatment. Detailed isolation results shown in Table 2. It is noticed calcium carbonate 1 to 2 % is an effective treatment for isolation of actinomycetes.

Isolation results of combination of dry heat and calcium carbonate treatment

A total of 33 actinomycetes strains were isolated from rhizosphere soil of Grape and wheat plant by a combination of dry heat (40°C) and various concentration of calcium carbonate of treatment. Detailed isolation results are shown in Table 3. It is noticed from the above results that the calcium carbonate 1 to 2% is an effective treatment for isolation of actinomycetes.

Table 1. Dry heat (°C) pretreatment

Sample	Dry heat Treatment	No. of Isolates
Rhizosphere soil of a grape plant	Dry heat 30	6
	Dry heat 40	13
	Dry heat 50	9
	Dry heat 60	3
	Dry heat 70	-
	Total Isolates	31
	Similar characteristics strains	24
	Total isolated strains	9
Rhizosphere soil of a wheat plant	Dry heat 30	7
	Dry heat 40	8
	Dry heat 50	5
	Dry heat 60	-
	Dry heat 70	-
	Total Isolates	20
	Similar characteristics strains	13
	Total isolated strains	7

Table 2. Calcium carbonate treatment and results

Sample	Calcium Carbonate Treatment	No. of Isolates
Rhizosphere soil of grape plant	0.25 %	6
	0.50 %	5
	1.0 %	6
	1.50 %	8
	2.0 %	8
	Total Isolates	33
	Similar characteristics strains	20
	Total isolated strains	13
Rhizosphere soil of wheat plant	0.25 %	3
	0.50 %	7
	1.0 %	7
	1.50 %	6
	2.0 %	6
	Total Isolates	29
	Similar characteristics strains	20
	Total isolated strains	9

Table 3. Dry heat and calcium carbonate treatment and results

Sample	Dry heat (40°C) and Calcium Carbonate Treatment	No. of Isolates
Rhizosphere soil of grape plant	0.25 %	5
	0.50 %	5
	1.0 %	8
	1.50 %	8
	2.0 %	6
	Total Isolates	32
	Similar characteristics strains	13
	Total isolated strains	19
Rhizosphere soil of wheat plant	0.25 %	2
	0.50 %	7
	1.0 %	8
	1.50 %	8
	2.0 %	3
	Total Isolates	28
	Similar characteristics strains	14
	Total isolated strains	14

Isolation results of phenol treatment

A total of 15 actinomycetes strains were isolated from rhizosphere soil of Grape and wheat plant by various concentrations of phenol treatment. Detailed isolation results are shown in Table 4. It is noticed the Phenol 0.5 to 1.5% is an effective treatment for isolation of actinomycetes.

Isolation results of dry heat and phenol treatment

A total of 17 actinomycetes strains were isolated from rhizosphere soil of Grape and wheat plant by various concentration of phenol treatment. Detailed isolation results are shown in Table 5. It is notice from the above results that the Phenol 0.5 to 1.5% is effective treatment for isolation of actinomycetes.

Isolation results of enrichment treatment

A total of 28 actinomycetes strains were isolated from rhizosphere soil of Grape and wheat plant by enriching in the starch casein broth with antibiotics. Detailed isolation results are shown in Table 6. It is noticed enrichment medium is favorable to grow actinomycetes, antibiotics in the medium is selective inhibition of bacterial as well as fungal growth.

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Screening of Actinomycetes for dipeptidyl peptidase-4 inhibitors production

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ABSTRACT

Hyperglycemia or high blood sugar is the most common cause of diabetes. Diabetes mellitus is the most common and fastest growing disease in the world. One of the therapies to treat diabetes is inhibition of dipeptidyl peptidase-4 inhibition by inhibitors. Dipeptidyl-peptidase 4 is a membrane glycoprotein having serine exopeptidase activity, which is cleaves X-proline or X-Alanine residue at N-terminus of peptides. Dipeptidyl peptidase-4 degrade glucagon-like peptide-1, which is the main cause for high blood glucose level, hence inhibitors of Dipeptidyl peptidase-4 have emerged as oral anti-diabetic agents. Microorganism, plant and chemical synthesis are sources of drugs. Actinomycetes are potential sources of enzyme inhibitors, drugs, amino acids, vitamins, pharmaceutical important chemicals etc. Present work mainly highlights the isolation of actinomycetes from soil samples and screening of extracts for dipeptidyl peptidase-4 inhibition activity. Isolation of actinomycetes was carried out by different methods and pretreatments. Isolated actinomycetes grown in fermentation condition and broth extracted with Isopropyl alcohol and ethyl acetate and obtain solid material after evaporation of solvents. The metabolites of each isolate were tested for inhibition of dipeptidyl peptidase-4 inhibition using spectroscopic method. Dipeptidyl peptidase-4 activity is measured by fluorescence of cleaved substrate, which is proportional to the enzymatic activity present. Total 130 actinomycetes strains were isolated. Among 130 extracts of actinomycetes, four extracts have shown positive results for dipeptidyl peptidase-4 inhibition. The actinomycetes strains that produce inhibitory compounds are A-9, A-12, C-4 and D-6. These results show that actinomycetes are a potential source for dipeptidyl peptidase-4 inhibitors, which may lead to valuable novel drugs for diabetic treatment.

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INTRODUCTION

Diabetes is a growing global health problem. Diabetes mellitus leads to cardiovascular disorders, muscular disorders and obesity etc. High blood sugar, or hyperglycemia, is a major concern, and can affect people with both type 1 and type 2 diabetes (Drucker, 2003). One of the approaches for treatment and control of diabetes is involved inhibition of dipeptidyl peptidase-4 (DPP4) by inhibitors (Lin *et al.*, 2019). DPP-4 is a membrane glycoprotein having serine exopeptidase activity (Holst, 2002). After ingestion of food,

gut releases incretins that gives signal for secretion insulin (Kieffer and Habener, 1999). Role of incretins, Glucagon Like Peptide-1 (GLP-1) and Gastric inhibitory peptide (GIP) in glucose homeostasis is depicted in Figure 1.

GLP-1 is inactivated by the DPP-4 enzyme and that affect on the insulation secretion and glucose metabolism (Zeng *et al.*, 2016). DPP-4 effect on blood glucose level has shown in Figure 2.

GLP-1 is inactive by the DPP-4 enzyme and hence it is no longer available for signaling to secretion of insulin (Drucker DJ et al.). Active GLP-1 and inactive GLP-1 and DDP action are shown in Figure 3.

New drug discovery approach is based on the design on the GLP-1 action and DPP 4 action (Wang *et al.*, 2017). There are two approaches for the drug discovery as 1. GLP-1 stabilization 2. DPP-4 inhibition (Ahrén, 2003). Approach for drug discovery and current marketed drugs are depicted in Figure 4 (Li *et al.*, 2018).

There are two sources of DPP-4 inhibitors; one is chemically synthesized and other natural source (Chandwad and Gutte, 2019). Natural source includes the microorganisms and plant (Singh *et al.*, 2017). Actinomycetes produces important bioactive compounds like enzymes, enzyme inhibitors, antibiotics, amino acid, anti-cancerous agents, antidiabetic drugs, anti-obesity drugs, and all of them have great economic importance (Imada, 2005). In literature several enzyme inhibitors producing actinomycetes are found from the different habitats including soil and marine environment (Raja *et al.*, 2010). Searches for drugs and other important chemicals have been remarkably successful and approximately two thirds of naturally occurring antibiotics have been isolated from actinomycetes.

There is a need of new and novel DDP-4 inhibitors, so it can lead to the development of novel drug for diabetes treatment. Hence present work mainly focused on the isolation of actinomycetes and screening for DPP-4 inhibitors, which may lead to development of new drug for obesity treatment.

MATERIALS AND METHODS

Soil sampling and processing

Five black soil samples were collected from different agricultural land. Soil samples were collected in a sterile plastic bag after removing 2-3 inch surface layer of soil by using sterile spatula (Mathew *et al.*, 2017).

Soil samples were air dried in laminar airflow unit for a day at room temperature and stored at 4°C until processed (Kumar and Jadeja, 2016).

Isolation of actinomycetes

Collected soil samples were pretreated at different conditions and isolation carried out on the selective medium with and without antibiotics. Combinations of physical and chemical methods were used for selective isolation of actinomycetes species.

Medium for actinomycetes isolation

Yeast Extracts Malt Extract Dextrose Agar (YMA) used and the same media with antibiotic Cycloheximide (50 μg/mL) and Nystatin (50 μg/mL) (Chandwad and Gutte, 2019).

Physical and chemical treatment combination (dry heat and calcium carbonate) and preservation of actinomycetes

One gram each of soil samples weighed in a flask and heat dried in oven at 35 to 45°C for 20 minutes. 1 gram dried sample was diluted to 9 ml by sterile normal saline water with 1 % calcium carbonate and incubated at 30°C for 72 hrs in incubator. Treated soil sample were centrifuged at room temperature at 4000 rpm for 4 minutes. The supernatant is serially diluted and each dilution plated on with and without antibiotics YMA medium using 100μl suspension. Plates were incubated at 28°C for 10 days. Dry and muddy characters of colony considered as actinomycetes which is cross checked by hyphal growth by using microscopy. Actinomycetes were subcultured by picking pure isolated colony on the YMA slants; slants incubated 28°C for 10 days, after full growth slants were stored at 4°C.

Shake flasks fermentation process

The grown culture on YMA slants scraped with 5 ml normal saline solution and 100 μl suspension inoculated in seed medium, 25 ml medium in 250 ml flask consists of soybean meal 1.5% (w/v), 2 % Dextrose, 1 % Glycerol, and flasks incubated at 30°C for 48 hrs at 200 rpm on rotary shaker. Grown culture 10 % used for 25 ml production medium inoculation. 25 ml production medium in 250 ml flasks consists of soybean meal 2.5%(w/v),0.5 % Yeast Extract,2 % Dextrose and1 % Glycerol. Production flasks incubated for 8 days at 28°C on a rotary sharer at 220 rpm (Imada, 2005).

Metabolites extraction with solvents

After completion of 8 days fermentation cycle, broth was harvested and stirred for 30 minutes to homogenize, and equal volume of Isopropyl alcohol (IPA) was added and stirred for 2 hrs. The solution was filtered through cloth and celite filter aid, and crud material obtained by evaporation of IPA under vacuum in Rota evaporator. Crude materials were

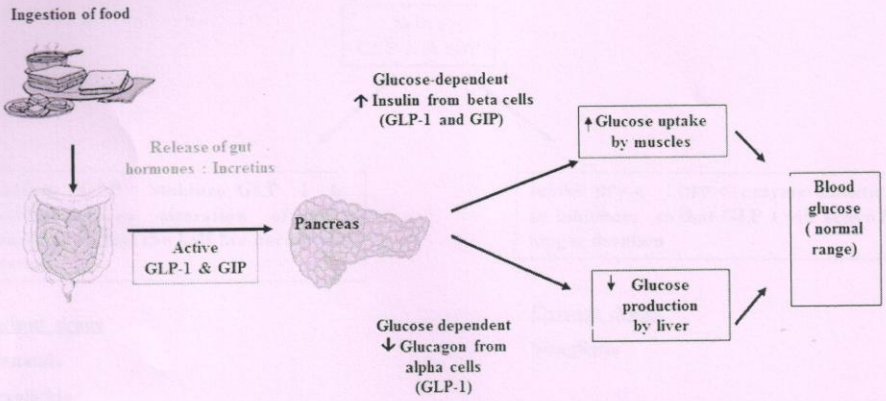


Figure 1: Role of Incretins in Glucose homeostasis

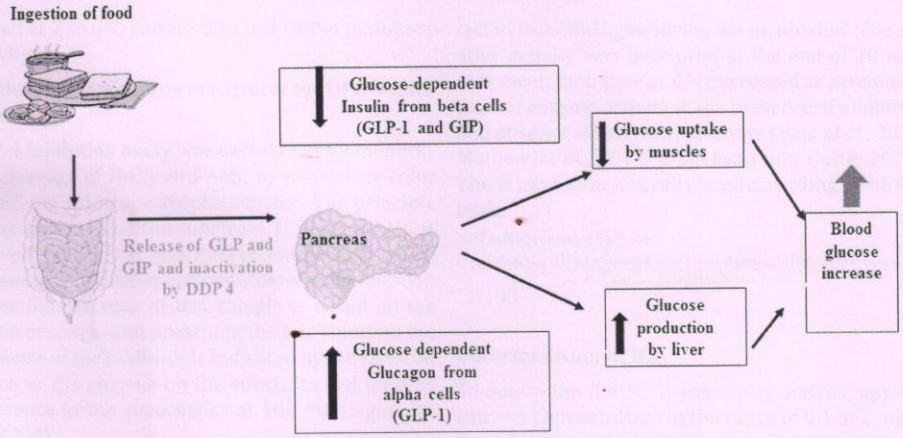


Figure 2: DPP-4 effect on blood glucose level

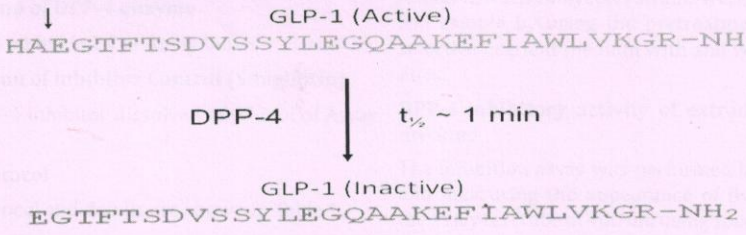


Figure 3: Active GLP-1 and inactive GLP-1 and DDP action

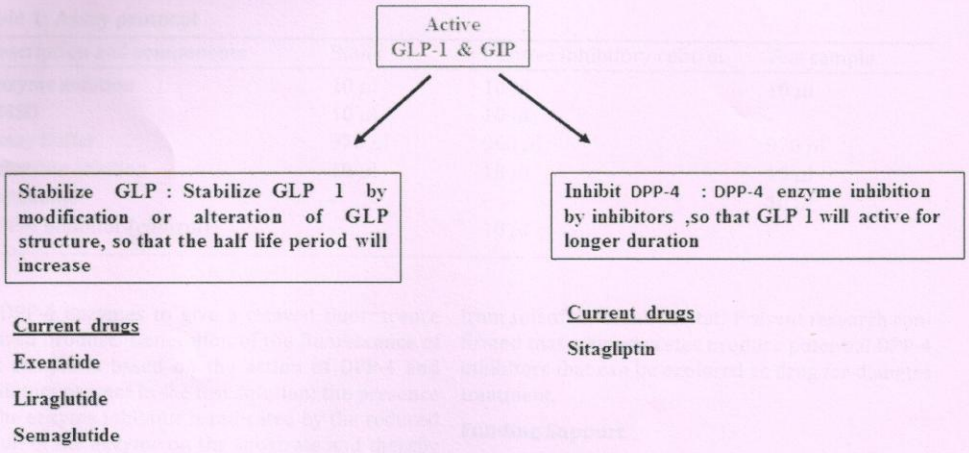


Figure 4: Current marketed drug and Approach

stored at 2 to 8°C and used to test DPP-4 inhibitory activity.

Preliminary screening of extracts for DPP-4 inhibition

DPP-4 inhibition assay was carried out by monitoring cleavage of H-Gly-Pro-AMC by measuring color at 460 nm using spectrophotometer. The principle of the assay is that the substrate, H-Gly-Pro-AMC is cleaved by DPP-4 to give fluorescent product which is measured by the spectrophotometer. Generation of the fluorescence of test sample is based on the action of compound present in the test solution; the presence of the inhibitor is indicated by the reduced action of the enzyme on the substrate and thereby difference in the absorbance at 460 nm (Sigma kit MAK203).

Preparation of metabolites solution

Dissolved 2 mg crude extract in 0.1 ml DMSO used for the assay inhibition assay.

Preparation of DPP-4 Substrate

2μl substrate added in 23 μl Assay buffer.

Preparation of DPP-4 enzyme

1 μl DPP-4 added in 24 μl Assay buffer.

Preparation of inhibitor Control (Sitagliptin)

2.5 μl DPP-4 inhibitor dissolved in 97.5 μl of Assay buffer.

Assay Protocol

Assay protocol and details are shown in Table 1.

Each blank, test and positive control reaction incubated for 10 minutes at 37 °C. Plates were protected from the light during the incubation. The relative activity was measured at the end of 10 minutes incubation time and is expressed as percentage ratio of enzyme activity in the presence of inhibitors and absence of enzyme inhibitors (Raja *et al.*, 2010; Mathew *et al.*, 2017; Chandwad and Gutte, 2019). The % inhibition was calculated according to the formula:

$$Inhibition\ (\%) = \frac{\triangle A_{460nm}\ (Uninhibited\ test) - \triangle A_{460nm}\ (Inhibited\ test)}{\triangle A_{460nm}\ (Uninhibited\ test)} \times 100$$

Determination of IC_{50}

To determine the 50 % inhibitory activity, applied extracts concentration in the range of 0.1 to 2 mg in the inhibitory assay. IC_{50} can easily determine on the graph plotting enzyme activity Vs extract concentration.

RESULTS AND DISCUSSION

Actinomycetes isolation results

A total 130 actinomycetes strains were isolated from soil sample by using the pretreatment and using selective nutrient medium with and without antibiotics.

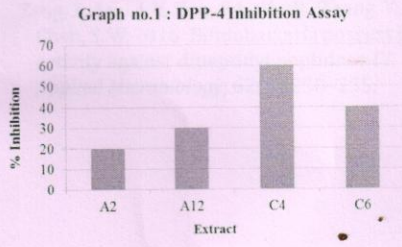
DPP-4 inhibitory activity of extracts of actinomycetes

The inhibition assay was performed by monitoring and measuring the appearance of fluorescence in the assay test tube at 460 nm using spectrophotometer after incubation for 10 minutes at 37°C. The principle of the assay is that the substrate is cleavaged

Table 1: Assay protocol

Description and components	Blank	Positive Inhibitory control	Test sample
Enzyme solution	10 μl	10 μl	10 μl
DMSO	10 μl	10 μl	-
Assay Buffer	970 μl	960 μl	970 μl
Substrate solution	10 μl	10 μl	10 μl
Metabolite	-	-	10 μl
DPP-4 inhibitor (control)	-	10 μl	-

by DPP-4 enzymes to give a cleaved fluorescence cleaved product. Generation of the fluorescence of test sample is based on the action of DPP-4 and inhibitors present in the test solution; the presence of the enzyme inhibitor is indicated by the reduced action of the enzyme on the substrate and thereby difference in the fluorescence of solution and intensity was measured at 460 nm in spectroscopy. The extract of isolate A9, A12, C4, C6, shown inhibitory activity as 20, 30, 60 and 40 % respectively. The extracts shown 20 to 60 % inhibition of DPP-4 are shown in the Graph 1.



Graph 1: Representation of % inhibition of DPP-4

DPP-4 inhibitory activity of inhibitors and IC_{50} determination

50 % inhibitory activity of A12, C4 and C6 of extracts determined using various concentrations of extracts in the assay. Extract of isolate A12, C4 and C6 having 50 % inhibitory activity at 2.5mg,1.4 mg and 1.8 mg respectively, whereas the Sitagliptin (standard) has shown 50 % inhibitory activity at below 0.1 mg concentration. Further investigation research is under progress on for fermentation process optimization and purification of compounds.

CONCLUSION

From the present study, it is noticed that actinomycetes found in soil are rich of bioactive compounds including enzyme inhibitors and such bioactive compounds and actinomycetes can be isolate from soil of different habitat. Present research confirmed that actinomycetes produce potential DPP-4 inhibitors that can be explored as drug for diabetes treatment.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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INTRODUCTION

Plant growth and development depend on the mineral and nutrients available in the soil. Plants face challenges in obtaining an adequate supply of these nutrients due to their relative immobility. Any nutrient deficiency results in decreased plant productivity, fertility, quality of flower and fruits. Nutrient deficiency has a significant impact on crop yield, plant, fruit and flower quality. Plant, flower and fruit quality is also depending on the other external factors such as plant pathogens including bacteria and fungi. Fruit quality of plant may get deteriorate due to bacterial and fungal infection. The fruits outer surface having bacterial and fungal load which are impacting on the fruit quality. It is well known that some microorganisms degrade fruit quality and some of microorganisms are beneficial to restore quality. This study considered isolation of actinomycetes from fruit samples and evaluating their bioactive ability which may play role in enhancing fruit quality.

Microorganisms constitute an in exhaustible reservoir of compounds with pharmacological, physiological, medical and agricultural applications [1-11]. Metabolites are produced by some organisms such as bacteria, fungi, plants and actinomycetes. Among the various groups of organisms that have the capacity to produce such metabolites, the actinomycetes occupy a prominent place. Actinomycetes are of universal occurrence in nature and are widely distributed in natural and man-made environments. They are found in large numbers in soils, fresh waters, lake, river bottoms, manures, composts and dust as well as on plant residues and food products. However, the diversity and distribution of actinomycetes that produce metabolites can be determined by different physical, chemical and geographical factors. Actinomycetes provide many important bioactive substances that have high commercial value [3-8]. The ability to produce a variety of bioactive substances have been utilized in a comprehensive series of researches in numerous institutional and industrial laboratories. Actinomycetes metabolites which have different biological activities, such as antibacterial, antifungal, ant-parasitic, anticancer, immunosuppressive actions, Antihyperglycemic and Antihyperlipidemic Agents. Streptomyces have prominent place and role in the actinomycetes group [3-8].

Very few research articles are published on the isolation of actinomycetes from fruits and screened for bioactive compounds. In various research papers it is observed that actinomycetes were isolated from the different plant parts including leaves, roots and fruits. Actinomycetes were isolated by researcher and tested for production of bio control agents and production of bioactive compounds [12,13].

Considering the limited number of bioactive compounds from actinomycetes isolates of plants and fruits, the attention is increased to isolate, screen more and more actinomycetes from different fruits. Actinomycetes strains that produces antibacterial, antifungal, antioxidant, enzyme inhibitory agents which may play an important role in plants growth and maintains of fruit quality.

MATERIALS AND METHODS

Fruit Sampling and Processing

Fruit of *Vitis vinifera L.* (Grape) and *Annona squamosa L.* (Custard apple) were collected from different locations. The outer layer of the fruit peeled and dried at 30°C for 48 hrs in laminar airflow unit and then stored at 4°C until processed.

Isolation of Actinomycetes

Different pre-treatments methods soil samples treatments and selective enrichment techniques with selective antibacterial agents used. Physical, chemical and combination of both methods were used for selective isolation of actinomycetes species [1-11].

Medium for Isolation of Actinomycetes

Yeast Extracts Malt Extract Dextrose Agar, Glycerol Asparagine Agar, and Starch Asparagine Agar were used and the same media with antibiotic Cycloheximide (50 µg/mL) and Nystatin (50 µg/mL) [7-11].

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ISOLATION OF ACTINOMYCETES FROM *Vitis vinifera* L. and *Annona squamosa* L. FRUITS AND SCREENING FOR BIOACTIVE COMPOUNDS

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ABSTRACT

Objectives: The current study focus and highlights on isolation of actinomycetes from *Vitis vinifera L.* and *Annona squamosa L.* fruits and screening for bioactive compounds being produced by actinomycetes particularly antibacterial activity and amylase inhibition activity and their role in fruit quality.

Methods: The actinomycetes were isolated from the fruit by pretreatment and growing on selective nutrient medium without and without antibiotics. Antimicrobial activity of extracts of isolates screened by antibiotic disc diffusion method and amylase inhibition activity screened using 3, 5-dinitrosalicylic acid by spectroscopic method.

Results: Total 16 actinomycetes were isolated from *Vitis vinifera L.* and *Annona squamosa L.* fruits sample by using calcium carbonate, dry heat pretreatment and grown on medium with and without antibiotics. Out of 16, 5 extracts of isolates shown antibacterial activity against *Escherichia coli* and *Pseudomonas aeruginosa*. Extracts of isolate G2 and C2 shown 30 and 50 % amylase inhibitory activity respectively.

Conclusion: The current study reveals that actinomycetes are present on the *Vitis vinifera L.* and *Annona squamosa L.* fruits, and they produces antibacterial activity which prevent undesired bacterial growth on fruit helps restore fruit quality. We found that isolated actinomycetes were source of bioactive compounds and amylase inhibition activity that help to prevent starch degradation.

Keywords: Actinomycetes; *Vitis vinifera L; Annona squamosa L*; antibacterial activity; amylase; antibiotics; zone of inhibition; inhibition assay.

Bacterial Strains

Escherichia coli (E. coli), Pseudomonas aeruginosa.

Treatment of fruit samples

Dry heat

One gram of samples was heat dried at 60°C for 30 minutes, sample was mixed in the 9 ml sterile water and centrifuged at 4000 RPM for 4 minutes, and the supernatant were serial was serial diluted and plated on the various medium.

Calcium Carbonate treatment

Dried sample was mixed with 1% Calcium carbonate in sterile plate and incubated at 30°C for 72 hrs. Treated samples were mixed in 9 ml distilled water and stored at static for 3 hrs. 1 ml supernatant was serially diluted and plated on various media plates. Plates are incubated at 28°C for 8 days.

Combination of dry heat and calcium carbonate treatment

One gram of fruit samples heat dried at 50°C for 30 minutes and treated with 1% calcium carbonate and incubated for 30°C for 72 hrs. The treated sample was centrifuged at 4000 RPM for 4 minutes, the supernatant was serially diluted and plated on the various media as described earlier and plates were incubated at 30°C for 8 days.

Preliminary Identification and Storage of Cultures

Colonies having muddy dry morphology considered as actinomycetes and cross checked by microscopic hyphal growth. Isolated strains grown on slants and stored at 4°C. Frozen culture prepared in 50% glycerol and stored in -20°C.

Fermentation Process and Extraction of Metabolites

Frozen culture gradually thawed to room temperature and 0.1 ml culture used for inoculation of 250 ml flask having 25 ml soybean casein digest medium with 2% dextrose as seed medium at 28°C on rotary shaker for 72 hrs. The well grown 10% seed inoculated in 250 ml flasks having 30 ml soybean casein digest medium with dextrose 2% and glycerol 2% as production medium at 28°C on rotary shaker for 240 hrs. Fermentation broth harvested after 240 hrs and metabolites are extracted with the solvents Isopropyl alcohol, acetone, acetonitrile and ethyl acetate. Crude metabolites obtained by evaporation of solvents by using evaporator.

Antibacterial Assay of Actinomycetes Extract

Antibacterial assay of extract was performed by disc diffusion method against *E. coli and P. aeruginosa*. Actinomycetes extracts concentration of 20 mg/ml (w/v) was prepared, and extract diffused on the disc. *E.coli and P. aeruginosa* were serially diluted and spread on the plate. The disc placed in the center of plate and plates kept for incubation 37°C for 24 hrs. The effectiveness of the extracts in tested bacterial strains was determined by measuring the zone of inhibition.

Amylase Inhibitory Activity of Extract. (3, 5-dinitrosalicylic Acid Assay (DNSA) to Test Amylase Inhibitory Activity [2]

The inhibition of enzyme was performed using DNSA method, enzyme inhibition assay mixture composed of 1ml of 0.02 M sodium phosphate buffer (pH 7.1 containing 6 mM sodium chloride), 1 ml of α- amylase enzyme (2% solution), and extracts at concentration of 2 mg/ml (w/v) were incubated at 37°C for 10 min. After pre-incubation, 1 ml of 1% (v/v) starch solution in the above buffer was added to each tube and incubated at 37°C for 15 min. The reaction was terminated with 1.0 ml DNSA reagent, placed in boiling water bath for 5 min, cooled down to room temperature, diluted, and the absorbance was measured at 540 nm. The control reaction representing 100% enzyme and did not contain any extract. Absorbance of Extracts without enzyme was also noted and subtracted. One unit of enzyme activity is defined as the amount of enzyme required to release one micromole of maltose from starch per min under the assay conditions.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Table 1. Pretreatment and isolation of actinomycetes results

Sample	Treatment	No. of Isolates	Observation
Grape fruit	Calcium carbonate	3	Fungal contamination observed
	Dry heat treatment	2	Fungal contamination observed
	Calcium carbonate and dry heat	3	Fungal contamination observed
	Calcium carbonate dry heat and antibiotic	6	No fungal contamination
	Number of isolates	6	Similar characteristics strains not counted
Custard apple	Calcium carbonate	3	Fungal contamination observed
	Dry heat treatment	2	Fungal contamination observed
	Calcium carbonate and dry heat	3	Fungal contamination observed
	Calcium carbonate dry heat and antibiotic	8	No fungal contamination
	Number of isolates	10	Similar characteristics strains not counted

Table 2. Diameter of zone of inhibition (mm) of extracts against *E.coli* and *P. auroginosa*

Isolates	Zone of inhibition against *E. coli*	Zone of inhibition against *P. aeuroginosa*
A3	9mm	8mm
A6	10mm	10mm
B1	10mm	10mm
B4	12mm	12mm
B9	12mm	12mm

The % inhibition was calculated according to the formula:

$$\text{Inhibition (\%)} = \frac{\Delta A540nm\ (\text{Uninhibited test}) - \Delta A540nm\ (\text{Inhibited test})}{\Delta A540nm\ (\text{Uninhibited test})} X\ 100$$

RESULTS AND DISCUSSION

Isolation of Actinomycetes

A total of 16 actinomycetes strains were isolated from grape and custard apple. It was observed that dry heat and calcium carbonate with selective antibiotics are suitable treatments for isolation of the actinomycetes I this study and details tabulated in the Table 1.

Antibacterial Assay of Actinomycetes Extract

The inhibition assay was performed using the DNSA method as described in the material and methods. The antibacterial property of the extracts tested against bacterial strains was determined by measuring the inhibitory zone. Out of 16, 5 extracts shown zone of inhibition against *E. coli.* And 6 extract shown inhibitory activity against *p. aeruginosa.* The study showed that extracts used in the study exhibited a varying degree of antibacterial activity against microorganisms tested (Table 2). Zone of inhibition against organisms are showed in Table 2.

Amylase Inhibitory Activity of Extract

16 extracts of isolates screened for alpha amylase inhibitors by DNSA spectroscopic method. Out of 16, 2 extract (G4 and C2) showed amylase inhibition, the inhibitory activity was 30 and 50% respectively.

CONCLUSION

From present study, it is observed that grapes and custard apple have actinomycetes load. It confirms from this study that actinomycetes found on the fruits produce antibacterial compounds. The present study concludes that antibiotics produced by actinomycetes are helping to protect fruit quality by inhibiting the bacterial growth on fruit. It also confirmed from the ongoing research that actinoycetes produce amylase inhibitors, which are preventing hydrolysis of starch. It can be said that the actinomycetes are beneficial for the grape and custard apple for restoration of the quality by inhibiting fruit water born pathogen *E. coli* and *P. aeruginosa*. Further this study concludes that extracts of isolates can explorer new bioactive compound as antibiotics, anitobesity and antidiabetes drug.