

Production of Myrosinase Enzyme Byactinomycetes Isolated from Cotton Soil

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Abstract— Myrosinase is defence related enzyme and capable of hydrolyzing glucosinilates into various compounds. It has immense potential in agricultural sector and cancer therapy. In the present study, myrosinase production by actinomycetes isolated from cotton soil has been studied. Myrosinase enzyme activity was assessed under different temperature regimes, pH range and incubation time using natural substrates as growth medium.

Key words: Myrosinase, actinomycetes, optimization

I. INTRODUCTION

MYROSINASE is a common name for the B-thioglycosidase, thioglucoside glucohydrolase, sinigrinase and sinigrase(1). The enzyme is responsible for the hydrolysis of glucosinolates, a group of sulphur-containing glucosides present in all members of cruciferae family including the brassica vegetables.

Applications

Myrosinase, (Thioglucoside glucohydrolase, sinigrinase, and sinigrase) is a family of enzymes involved in plant defense against herbivores. These molecules are useful, not only for their activity against bacteria, fungi, nematodes, tumour cell growth and in cancer prevention (2), but also has the potential to be used as intermediates in chemical synthesis (3). Myrosinase was not only found in plants but also in micro organisms.

Myrosinase glucosinolate as biofumigant against pests

The glucosinolate myrosinase system has been investigated as a possible biofumigant to protect crops against pests. The potent glucosinolate hydrolysis products (GHPs) could be sprayed onto crops to deter herbivory(4).

Anticancer activity

In recent years, there has been a massive increase in interest in glycosidases. Inhibition of the enzymes that process carbohydrates has potential therapeutic applications for the treatment of cancer, AIDS and other viral infections(5).

Even though many studies were available on myrosinase production by plants, very little information was available on microbial production of myrosinase enzyme, especially actinomycetes. Hence the present study aims in

OBJECTIVES

1. Studying myrosinase production from actinomycetes isolated from the cotton soil using sinigrin barium agar technique.
2. Myrosinase production by solid state fermentation using natural substrates.

3. Actinomycetes strain improvement by mutagenesis to enhance myrosinase production and optimization of pH, temperature and incubation time.

II. MATERIALS AND METHODS

Collection of soil sample

The soil sample was collected from the cotton field, which is present in village Palaparru, Guntur District at various places to a depth of 5 cm. It was selected to obtain range of texture, pH and organic matter.

Media Preparation

The culture medium is prepared for the 3 types of organisms. For the Actinomycetes Glycine Glycerol Agar medium, for the fungi Czapeck Dox agar medium and for the bacteria Nutrient Agar medium was prepared with the following composition. Required serial dilutions for the different types of organisms isolation were as follows

Bacteria - 10^{-6} , 10^{-7} , 10^{-8}

Fungi - 10^{-5} , 10^{-6} , 10^{-7}

Actinomycetes - 10^{-4} , 10^{-5} , 10^{-6}

The glucosinolate used as a substrates was, sinigrin for standard (Sigma chemical co.st.Louis.mo) cauliflower, cabbage, turnip, raddish.. Toluene was obtained as a Fisher certified reagent (Fisher scientific co. 1L).

Screening for myrosinase production

The selected strains were inoculated onto sinigrin-barium agar plates, and incubated at 30°C. Growth was observed daily, and the strains that grew on sinigrin-barium agar plates were re-plated onto mustard extract agar (6). Incubation was carried out at 30°C. The plates were observed daily. Each distinct growing colony was picked and then plated onto nutrient agar or potato dextrose agar plates. Myrosinase production by the selected strains was tested

Assay :- (standard procedure)

Absorbance of the pink color of the quinoneimine complex formed was measured with a spectrophotometer adjusted to a wavelength of 505nm. Graph was constructed with the values obtained and concentration of glucose was calculated based on the standard curve.

Proper controls must be performed in each series of analysis to measure glucose background in soil. To perform controls 2.3 ml of TES buffer (pH7) and 0.2ml of toluene were added to 1ml of culture to obtain a final concentration of 200mM (7).

Testing with different natural substrates

The above assay is performed by using different types of substrates. The substrates are 5 types. They are seed extract of mustard, cauliflower, cabbage, turnip, raddish. The substrates

are prepared by taking 0.5ml prepared in 0.1 m TES buffers pH7(8).

Preparation of the different extract mediums :

Natural extract medium

Natural extract culture medium was prepared by diluting the mustard extract Cauliflower, cabbage, turnip, raddish to final glucosinolate concentration of 10 mM in 0.1 m potassium phosphate buffer, pH 6.5. The medium was autoclaved at 121°C for 15 minutes. Cell free extract obtained after centrifugation (12000 rpm 25 minutes), was assayed for myrosinase activity content.

Mutagenesis

Ten milliliters of actinomycetes suspension was placed into a sterile petri dish of diameter 9 cm and irradiated with UV light at a distance of 30 cm away from the light source (254 nm, 60w) for various times ranging from 0-30 minutes. Duplicates were maintained for each interval. After treatment with UV, the plates were incubated in the dark at 30°C for 3 – 7 days.

Optimization of temperature, pH and incubation time :

The optimization of cultural conditions for myrosinase production by actinomycetes mutants was carried out in extract medium. (mustard seed, cauliflower, cabbage, turnip, Raddish) with various conditions such as pH, incubation time and incubation temperature(9).

The effect of incubation temperature on myrosinase production was studied at 20°C to 60°C. Here we maintained various conditions such as pH 4, 5, 6, 7, 8 and incubation time is 0, 5, 10, 20, 30 minutes.

Identification of actinomycetes

Morphological identification by staining technique

The selected actinomycetes was subjected to the standard gram staining technique to know the morphology and arrangement of cells. Endospore staining using Malachite green and safranin is done to reveal whether it is a spore forming or non spore forming. If positive, arrangement of spores.

Identification based on cultural characterization

Colony morphology of the isolate in terms of size, shape, colour, margin, appearance, elevation are observed for culture based identification of the culture.

Biochemical identification

Biochemical characterization of the isolates was carried out by performing various biochemical tests by adopting standard methods (10,16). Tests were performed in the presence of respective positive and negative controls.

Molecular characterization of the isolate was carried at Credora life Sciences, Bangalore which involves the following steps

1. Preparation of genomic DNA
2. Amplification of 16S rRNA sequence by PCR
3. Sequencing of 16S rRNA

Sequences obtained were further used for BLAST analysis from the NCBI data base to obtain the sequence similarity with related organisms. The sequences were multiple aligned with representative sequences of selected genera using CLUSTALW program. The phylogenetic tree for the datasets was utilized to access the relationship between the

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III. RESULTS

Isolation of microorganisms from cotton soil

Three black soil samples were obtained from the selected cotton field and physico chemical properties are analysed (Table 1). Majority of the isolates obtained are bacteria, followed by actinomycetes and fungi. Details of the microbial isolates obtained and their cultural characteristics are represented in table 2 and figure 1

TABLE 1
PHYSICO CHEMICAL PROPERTIES OF THE BLACK COTTON SOIL

Parameter	Values
Sand	70
Silt	11
Clay	5
pH	8.46
Water holding capacity	0.4
Organic Matter ^a Walkley Black method(Jackson, 1971)	0.44
Total nitrogen ^b MicroKjeldahlMethod(Jackson,1971)	0.04

TABLE 2
COLONY MORPHOLOGY OF THE ISOLATED MICROORGANISMS FROM COTTON SOIL

Colony Morphology	Bacteria	Fungi	Actinomycetes
Size	0.5cm	0.5-1cm	1-2.5cm
Colour	Pale and cream	Pale brown	Maroon
Shape	Irregular	Irregular	Irregular up and down
Elevation	Flat	Low convex	Convex
Margin	Undulate	Entire	Erose
Appearance	Rough	Smooth	Smooth
Pigmentation	Greenish brown	Light Green	Golden Yellow
No. of colonies	180	70	90

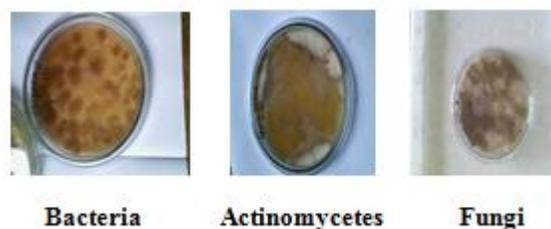


Fig. 1. Colony morphology of microbes isolated from cotton soil

Screening for myrosinase enzyme production by sinigrin barium agar plate technique The screening result showed all the three types of microorganisms with positive opaque zone on sinigrin-bariumsulphate agar



Myrosinase assay: The myrosinase enzyme activity measured in terms of glucosinolates liberated in detected by using TES buffer. All the isolates are positive result for the myrosinase production by the appearance of pink colour (Table 3)

TABLE 3
MYROSINASE ENZYME ACTIVITY OF THE ISOLATES

Sinigrin substrate	505 nm	Wave	Length	
Organism O.D Values	Control 0.098	Bacteria 0.2563	Fungi 0.0945	Actinomycetes 1.5185

Production of myrosinase enzyme using natural substrates by solid state fermentation Five different natural substrates (mustard seed, cabbage, cauliflower, turnip, radish) have been tested for enzyme production with actinomycetes only as it showed maximum value in assay. Mustard seed followed by cauliflower showed high enzyme activity with actinomycetes indicated in table 4 and figure 3. Minimum activity was noted in radish and cabbage.

TABLE 4
MYROSINASE PRODUCTION WITH DIFFERENT NATURAL SUBSTRATES

Substrate	Value in units / ml
Control	0.098
Mustard seed	3.30
Cauliflower	3.21
Cabbage	2.24
Turnip	2.52
Radish	2.83

Strain improvement of actinomycetes culture for enhancement in enzyme production

The values obtained after mutagenesis are given in table 5. There was an increase in the value from initial 1.5185 to 2.25 after mutagenesis. Change in appearance of the actinomycetes colony slightly and increase in number of colonies was noticed as evident from table 6 and figure 4. Whereas bacterial colonies decreased in number tremendously and fungi also followed same trend as that of actinomycetes. Mutagenic actinomycetes was designated as PAM for convenience.

TABLE 6
COLONY MORPHOLOGY OF THE ISOLATED MICROORGANISMS FROM COTTON SOIL AFTER MUTAGENESIS.

Sinigrin substrate	Value obtained after mutagenesis
Control	0.094
Actinomycetes	2.25

Colony Morphology	Actinomycetes(PAM)
Size	1-2.5 cm
Colour	Maroon
Shape	Irregular and round
Elevation	Convex
Margin	Erose
Appearance	Smooth
Pigmentation	Golden Yellow
No. of colonies	120

Myrosinase production by solid state fermentation with natural substrates after mutagenesis

The experiment was conducted similar to that of non mutagenic cultures and the results were shown in table 7.

The activity was enhanced in all the five samples with maximum in mustard seed extract as observed previously

TABLE 7
SOLID STATE FERMENTATIVE PRODUCTION OF MYROSINASE WITH NATURAL SUBSTRATES USING MUTAGENIC ACTINOMYCETES STRAIN

Substrate	Enzyme activity
Mustard seed	4.50
Cauliflower	3.91
Cabbage	3.25
Turnip	2.97
Radish	3.56

Effect of pH, temperature and incubation time on myrosinase in natural extract medium

The influence of the above three parameters has been studied in mutagenic strain of actinomycetes (PAM) in all the natural substrates and reported in table 8. With mustard medium, optimum pH is 5 followed by 6 and 4. Unlike mustard medium, maximum value is at neutral Ph followed by 8 Ph. In radish extract medium, the values were less but followed the same trend of maximum value at neutral pH. In cabbage, no significant effect was found but however ph 8 is maximum. Similar is the case with turnip extract medium.

TABLE 8
EFFECT OF PH WITH FIVE NATURAL SUBSTRATES MEDIUM ON MYROSINASE

Mustard seed extract medium	Cauliflower extract medium	Cabbage extract medium	Turnip extract medium	Radish extract medium	Substrate
1.5567	1.2203	0.1232	0.1720	0.1720	pH 4
1.837	1.2660	0.1586	0.2520	0.2520	5
1.663	1.2121	0.1432	0.2635	0.2635	6
1.2082	1.9202	0.1605	0.3483	0.3961	7
1.2275	1.8601	0.2215	0.3599	0.3599	8

Effect of temperature with five natural substrates medium on myrosinase

Myrosinase activity was more at 20°C with mustard and again there is a slight decrease at 60°C. But at 40°C, decline was observed. Whereas maximum value was obtained at 40°C with cauliflower medium and radish extract medium. With cabbage, it is noticed at 60°C. No significant change was seen with turnip.

TABLE 9
EFFECT OF TEMPERATURE WITH FIVE NATURAL SUBSTRATES MEDIUM ON MYROSINASE

Substrate	Mustard seed extract medium	Cauliflower extract medium	Cabbage extract medium	Turnip extract medium	Radish extract medium
20°C	1.7089	1.1570	0.1232	0.4991	0.1899
30°C	1.5056	1.0727	0.1586	0.3977	0.1572
40°C	1.4043	1.5469	0.1432	0.4644	0.2206
50°C	1.5086	1.1784	0.1605	0.3881	0.1881
60°C	1.6076	1.1789	0.2215	0.3644	0.1862

Effect of incubation time with five natural substrates medium on myrosinase

Highest values were observed at 5 mins followed by 10 mins and 30 mins with all the five mediums.

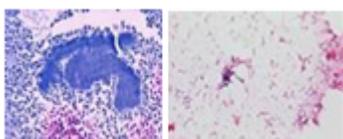
TABLE 10
EFFECT OF INCUBATION TIME WITH FIVE NATURAL SUBSTRATES MEDIUM ON MYROSINASE

Substrate	Mustard seed extract medium	Cauliflower extract medium	Cabbage extract medium	Turnip extract medium	Radish extract medium
0 mins	1.5056	1.1570	0.2252	0.4991	0.1899
5 mins	1.7089	1.0727	0.5469	0.3977	0.1572
10 mins	1.4043	1.5469	0.3991	0.4644	0.2206
20 mins	1.5086	1.1784	0.2818	0.3881	0.1881
30 mins	1.4076	1.0745	0.5515	0.4474	0.1662

Identification of the actinomycetes isolate
Morphological

TABLE 11
MORPHOLOGICAL CHARACTERISTICS OF THE ISOLATE

Gram staining	Spore staining	Arrangement
Gram positive	No spore forming	Bacilli in singles

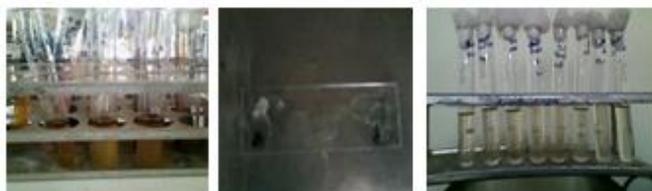


Biochemical Characterization

Fig. 3. Gram staining Spore staining

Biochemical tests were performed to characterize the isolates. The results for citrate test, urease test, Nitrate test, methyle Red test, VP test, Indole test, Oxidase test, Catalase test, Sugar fermentation test, (Fig.1-Fig.9) were presented in the following table

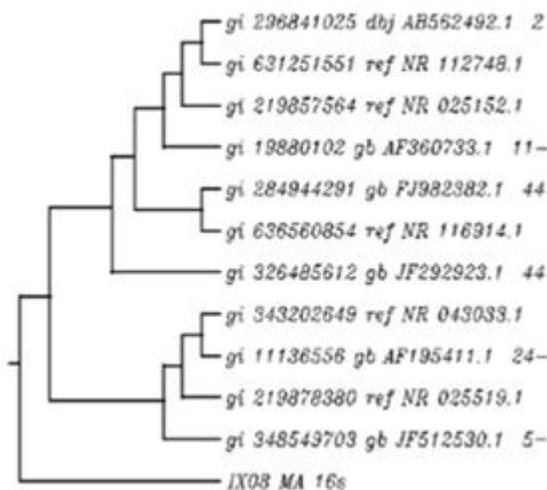
Biochemical Tests	Soil Bacterial Isolates		
	Bacteria	Actinomycetes	Fungi
Citrate test	-ve	+ve	+ve
Urease test	+ve	-ve	+ve
Nitrate test	+ve	-ve	-ve
Methyl Red test	+ve	-ve	-ve
VP test	-ve	-ve	-ve
Indole	+ve	+ve	+ve
Oxidase test	+ve	-ve	+ve
Catalase test	+ve	-ve	-ve
Sugar fermentation test	-ve	-ve	-ve



Molecular identification

The NCBI BLAST search program showed that the sequence data of isolate PAM, which showed positive

response to myrosinase production was also subjected to 16s rRNA sequencing and identified as actinobacterial member which has 97% homology with N0cardiopsis potens.



IV. DISCUSSION

Soil sample is suspected to contain myrosinase producing microorganisms were collected. Here growth was observed, a decrease in the sinigrin and other content was observed for only for interesting strains with high activity. The actinomycetes possessed that most promossing myrosinase activity and was , therefore, selected for all further experiments. Assessment of myrosinase activity is based on measuring glucose released when soil is incubated with the substrates like sinigrin, cauliflower, cauliflower, Turnip, Raddish. The activity of enzyme in air-dried versus fresh soil and the effect of buffer pH, amount of soil, time of incubation and temperature were all evaluated to optimize and characterize myrosinase activity in soil. Unless indicated otherwise, all resulted presented were obtained using the standard assay.

Glucose is water soluble and can be readily extracted from soil. Extraction efficiencies were always 100±0.9% when glucose was added to soil and then immediately extracted, however, when soil amended glucose concentration were incubated for 4 hours, extraction efficiency was greatly reduced to as low with comparision of control before mutagenesis in the media sinigrin was 0.094 (0.093 control) in the fungi. And high extraction efficiency is seen in Actinmycetes of sinigrin is 1.5185 (0.098 control). In the overall 3 medias the enzyme activity high by the actinomycetes.

The survivors of UV mutagenesis of wild-type mutants were selected for myrosinase hyperproduction by observing on Glycine Glycerol agar medium growth profiles on mustard extracted media, activity and stability of enzyme. The Result of mutagenesis gave mutant trains. The mutants with sufficiently high stability was the strain of choice due to its highest myrosinase production comparing to other mutants. Here grow 3 types of organisms grown on mustard extract medium with no supplement gave relatively high myrosinase activity compare to the before mutagenesis(11). To our

knowledge myrosinase – over producing mutants characterized by such high levels of enzymatic activities have not been previously reported. The advantages of higher myrosinase activity and ability to grow in the low cost medium.

The stability of enzyme present in the cell free extracts was subsequently determined. Myrosinase from the wild-type actinomycetes was not so stable that it was inactive after 2 h at 30°C. The stability of the wild-type enzyme was similar to those of enzymes from other micro-organisms suggesting myrosinase of microorganisms were not stable at high temperature.

UV mutagenesis was able to increase myrosinase stability. The myrosinase from the mutants reported here remained stable at 4°C for 2 weeks (12).

The product analysis of myrosinase from wild type organism and mutant strains. Revealed that glucosinolate was degraded to allylthiocyanate in neutral condition, while allyl cyanide could not be detected. The glucosinolate found in mustard seed cake (*Brassica Juncea* var. *forge*) was sinigrin. This Result was similar to other previous reports. The types of products obtained from glucosinolate degradation are depending on source and type of glucosinolate and pH of enzyme reaction. This results as useful for allylthiocyanate production and other products from glucosinolate hydrolysis.

The mutant strain degraded higher glucosinolate concentration (10 mm) at the same time course of growth in liquid media. Glucosinolate concentration decreased rapidly in the beginning of the cultivation. The growth profit has small amounts of glucose liberated to the culture media. In this case, glucose should be liberated from some other polysaccharides, not the glucosinolate. It was previously proposed that Brassica oilseeds contain variable amount of polysaccharides. (3,4) has confirmed that mustard seed meal (*Brassica juncea*) contained 21.3% carbohydrates. These polysaccharides may serve as substrates of glucoamylase from wild type and mutant strains as observed in this study by a release of glucose into the culture medium.

In conclusion, a myrosinase – overproducing mutant, Actinomycetes has been obtained and characterized to provide the enzymes with high stability, high production valuable hydrolysis products and constant production stability while maintaining a physiological behaviour similar to that of the wild-type strain (13,14). Hence in our opinion, this mutant enzyme offers a great potential for industrial applications for example feeds detoxification and enhanced yield of flavour production (allylthiocyanate production).

The Actinomycetes are gram positive, filamentous bacteria intermediate in properties between true bacteria and fungi. Like bacteria, they possess cell wall containing muramic acid, prokaryotic nuclei and are susceptible to antibacterial antibiotics, whereas like fungi they form a mycelial network of branching filaments. The actinomycetes are true bacteria with a superficial resemblance to fungi.

They are related to corynebacterium and mycobacterium. They are non-motile, non-sporing, non-capsulated filaments that break up into small bacterial fragments and live freely in nature, in water and particularly in the soil. The family actinomycetes contains three major medically important

genera, Actinomyces, Nocardia and Actinomadura. Another genus, streptomyces rarely causes disease in man, but its medial importance lies in the production of antibiotics by its several species. In addition, inhalation of some thermophilic actinomycetes such as micropolyspora faeni and thermoactinomyces sp. May cause allergic alveolitis (farmers lung and bagassosis). Actinomyces is anaerobic or microaerophilic and non-acid-fast. While Nocardia is acid-fast and aerobic. Streptomyces and Actinomadura are non-acid fast and aerobic.

The actinomycetes sps using the nitrogen source is glucosinolate extracts like mustard seed extract or natural media & cabbage, cauliflower, turnip, Raddish extracts., used to produce myrosinase enzyme (15). After incubation, Assay enzyme is released. This enzyme is isolated and to estimate the concentration of enzyme present by using the standard procedure. The extra cellular enzyme is estimated by Assay.

In this method to study the effect of pH and temperature and incubation time, on myrosinase activity on soils. TES buffer was adjusted to range from pH toluene (0.2 ml) and extract is prepared in the buffer they are added to the sample incubated 4 hours and activity is measured. Has added silver nitrate in last step in order to stop activity of all enzyme. Measure the absorbance of the pink colour of the Quinolone complex formed with a spectrophotometer and adjusted to wavelength of 505 nm (17).

The temperature, incubation time, and pH shown significant effect on myrosinase enzyme activity. The myrosinase enzyme activity at temperature 20°C of incubation time 20. The pH at 7 for incubation time is 5 and 10 minutes. How stimulatory as well as inhibitory effect on enzyme activity and the dry weight is also calculated (18). The highest dry weight is at mustard seed extract medium at pH 4,8,7 and time incubation is 30 minutes of dry weight is 0.06 gms.

V. CONCLUSIONS

Myrosinase production is observed in maroon colour Actinomycetes present in soil where there is growth of brassica and sinapis plants. Hydrolysis of glucosinolate (Sinigrin) produces detectable amounts of D-glucose that can be colorimetrically measured and used as a quantitative measure or myrosinase assay in soils.

Maximum activity in soils is achieved with mustard seed extract medium at pH 7, 20°C temperature and 20 mins incubation time.

Myrosinase activity was further enhanced in mutagenic strain compared to the non mutagenic culture of actinomycetes.

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