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Research Article

Isolation and Screening of Multivariant Enzyme Producing Endophytic Bacteria from *Citrus limon*

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Abstract

Endophytes have a property of various enzymes production which work as Plant Growth Promoting Bacteria (PGPB) and are a good source of secondary metabolites with phytochemicals properties. The objective of this study was to identify the enzymatic properties of the endophytes which were isolated from various part of *Citrus limon* plant. A total of 27 bacterial endophytes were isolated with three Bacillus spp. gave best results and showed multivariant in enzymes production. It may be good for plant health and growth.

Keywords: *Bacillus* Spp.; *Citrus limon;* Endophytic Bacteria; Enzymatic Properties; Multivariant

Introduction

PGPR decorate plant growth by means of direct and oblique manner, but the specific mechanisms for booster have not all been nicely characterized. PGPR that not directly decorate plant growth via suppression of phytopathogens accomplish that by an expansion of mechanisms [6]. These consist of the capacity to supply siderophores that chelate iron, making it unavailable to pathogens; the capability to synthesize anti-fungal metabolites including antibiotics, fungal cellular wall-lysing enzymes, or hydrogen cyanide, which suppress the growth of fungal pathogens; the capacity to efficaciously compete with pathogens for vitamins or particular niches on the root; and the potential to set off systemic resistance [7].

In the prevailing take a look at, we provide evidence that the *Citrus limon* have the various residences and because of the presence of their microflora, Endophytes having the numerous enzymes houses. Earlier many studies reported that Amylase, Proteas, Cellulase, Caseinase, Urease and Dehydrogenase all this enzymes production seen by different organism, Study shows that these endophytes produced enzymes more than one and as it may be useful for human as well as plant health too. We additionally demonstrate that the reactive hexose(s) multivariant enzymatic microorganism also can be as it should be determined the use of this method.

Materials and Methods

Culture collection and isolation

The whole *Citrus limon* plant as a test sample was collected from Scientific Nursery of Department of Horticulture, Anand agriculture University, AAU, Anand, Gujarat, India. Total twenty-seven bacterial endophytes were isolated from stem, root, leaf and seed of *Citrus limon.* They were characterised for Morphological, cultural and Biochemical characteristics on the basis of Bergey's manual of systematic bacteriology [8]. All bacterial cultures were cultivated on Nutrient agar media and also preserved at 4°C on Nutrient agar plate. For all the enzymatic tests, 24h activated culture suspension was prepared in 10 ml of sterile distilled water. These were then inoculated in various medium as per multiple enzymatic activities.

Starch hydrolysis test

All twenty-seven isolates as test culture were inoculated on starch agar plate as spots and incubated at 37˚C for 24h. These were observed for transparent zone surrounding the colony. The plates were flooded with Lugol's iodine and immediately read, because the blue colour fades rapidly [4].

Casein hydrolysis test

All isolates were inoculated on skim milk agar plate as line and incubated at 37˚C for 24h [4].

Urea hydrolysis test

Inoculated all test culture on Stuart's urea broth/plate and incubated at 37˚C for 24h [4].

Dehydrogenase test

The test involves the detection of the dehydrogenase enzyme by using methylene blue as the compound which accepts the hydrogen released in the respiratory electron transport chain, and gets reduced (colourless form). Activated dense culture (~10^6 organisms/ml) spot inoculated on sterile nutrient agar containing 10% sterile methylene blue, incubated at 37˚C for 24h [4].

Lipid hydrolysis test

All test cultures were inoculated on the Tributyrene agar plates as line and incubated at 37˚C for 24h. The plates were Observed for the clear zone of calcium carbonate solubilization and the change of colour surrounding growth of organisms [4].

Cellulase production

Cellulose agar plates were prepared using the CMC as a carbon source was stroked on the solidified agar and allowed to incubate for 48 hr to express cellulose depolymerization through cellulase production into its surrounding medium. The plate was stained with 0.1% Congo red and counterstained with 1M NaCl for 15-20 min. the zone of cellulose hydrolysis appeared as a clear area against the Congo red stain background. The cultures were grown under optimum condition for cellulase production [6].

Result and Discussion

Bacterial isolates

A total of 27 bacterial isolates were obtained from *Citrus limon* plant, out of them 8 bacteria isolated from the root which were coded as MSZR series, 8 bacteria isolated from steam which were coded as MSZS series, 10 bacterial isolates from leaf which were coded as MSZL series and one bacterial isolated encoded MSZPse from seed of *Citrus limon.* Their Morphology and gram reaction were also studied (Table 1).

Amylase production

Organisms producing amylase utilize starch in the vicinity of the colony, hence when the medium is flooded with iodine solution; colourless zone is seen surrounding the colony, the remaining portion of the medium turns blue. When the starch is added in hot water, amylose fraction being soluble diffused in it; while amylopectin remains insoluble. As a result, milky white colloidal solution is formed. Hence the starch agar plate has a opaque, milky white appearance. If the organisms growing on such a medium produce amylase, the starch from the vicinity of the colony is utilized giving a clear transparent zone surrounded by opaque medium. Maximum zone of hydrolysis observed in MSZLe and MSZPse, then MSZLd and MSZLc (Table 1) (Figure 1 and Figure 3).

Figure 1: Amylase producing bacteria shows zone of starch hydrolysis.

Protease and caseinase production

Casein is sparingly soluble in water and hence medium formed due to its incorporation is opaque (milky white). Caseolytic organisms produce casease which hydrolyse casein to soluble form para-

casein. Hence the clear zone is observed in the medium surrounding the growth. Maximum zone of skim milk utilization observed in MSZPse and MSZLe then MSZLd and MSZLc then MSZLs and MSZL5 (Table 1) (Figure 3).

Urease production

Development of pink colour in urea broth indicates positive test indicating bacteria utilize the urea and broth change yellow to pink in colour. Maximum enzyme shown in MSZLd and MSZLe then MSZLc and MSZPse then MSZRc and MSZR1 (Table 1).

Dehydrogenase production

The test involves the detection of the dehydrogenase enzyme by using methylene blue as the compound which accepts the hydrogen released in the respiratory electron transport chain, and gets reduced (colourless form). All isolates shows positive of dehydrogenase enzymes out of them Maximum zone of Methyl decolourize shown in MSZRc and MSZSa then MSZR2 and MSZS2 then MSZS5 and MSZLc (Table 1) (Figure 2 and 3).

Sr. No.	Isolates	Morphology	Gram reaction	Amylase	Caseinase	Lipase	Cellulase	Urease	Dehydrogenase
1	MSZRa	Cocci	Gram +ve	+	+	-	-	-	-
2	MSZRb	Cocci	Gram +ve	+	-	-	-	-	++
3	MSZRc	Bacilli	Gram +ve	-	-	-	-	+	+++
4	MSZRd	Cocci	Gram +ve	-	-	-	-	-	+++
5	MSZR1	Cocci	Gram +ve	+	-	-	-	+	-
6	MSZR2	Cocci	Gram +ve	+	-	-	-	-	+++
7	MSZR3	Cocci	Gram +ve	++	+	-	-	-	+++
8	MSZR4	Cocci	Gram +ve	+	-	-	-	-	+++
9	MSZSa	Bacilli	Gram +ve	++	++	-	+	-	+++
10	MSZSb	Short rod	Gram -ve	++	+	-	-	+	++
11	MSZSc	Cocci	Gram -ve	-	-	-	-	-	++
12	MSZS1	Cocci	Gram +ve	+	-	-	-	-	++
13	MSZS2	Cocci	Gram +ve	-	-	-	-	-	+++
14	MSZS3	Cocci	Gram +ve	-	-	-	-	+	++
15	MSZS4	Cocci	Gram +ve	-	+	-	-	-	-
16	MSZS5	Cocci	Gram +ve	+	++	-	-	+	+++
17	MSZLa	Cocci	Gram -ve	-	-	-	-	+	+++
18	MSZLb	Cocci	Gram +ve	+	-	-	-	+	+++
19	MSZLc	Bacilli	Gram +ve	++	++	+	++	+	+++
20	MSZLd	Bacilli	Gram +ve	++	++	++	+	+++	++
21	MSZLe	Bacilli	Gram +ve	+++	+++	+++	++	++	+
22	MSZL1	Cocci	Gram +ve	+	+	-	-	-	++
23	MSZL2	Cocci	Gram +ve	+	-	-	-	-	+++
24	MSZL3	Cocci	Gram +ve	+	+	-	-	-	+
25	MSZL4	Cocci	Gram +ve	+	-	-	-	-	+
26	MSZL5	Cocci	Gram +ve	+	-	-	-	-	+++
27	MSZPse	Bacilli	Gram +ve	+++	+++	++	+++	+	++

Table 1: The result of the Enzyme activity assay of Endophytic bacteria isolated from *Citrus limon.*

Key: + = Poor Growth; ++= Moderate Growth; +++ = Optimum Growth; - = No Growth Observed

Figure 2: Dehydrogenase enzyme producing bacteria shows zone of methyl decolourization.

Lipase production

Tributyric acid (Tributyrene) is commonly used to check the lipase activity by microorganisms. Tributyrene is hydrolysed as follows:

$$\text{Tributyrene} + 3H_2O \xrightarrow{\text{lipases}} \text{Glycerol} + \text{Butyric acid}$$

The butyric acid so formed reacts with calcium carbonate to form solubilised calcium butyrate:

$$CH_3COOH + CaCO_3 \longrightarrow CH_3COOCa + CO_2$$

By adding a pH indicator to the culture medium, it is also possible to detect the hydrolysis to lipids by the colour change. For example, Nile Blue Sulphite has lavender colour and it turns royal blue around lipolytic bacterial colonies due to the acid pH. Lipase enzyme production observed only in MSZL MSZLe > MSZPse > MSZLc > MSZLd (Table 1).

Cellulase production

Cellulase production was checked in solid media [4]. CMC was used as a substrate and maximum enzyme activity was found at 48h in MSZPse and MSZLe respectively and after 5 days in MSZLc > MSZLd > MSZSa. similar results were observed with *T. reesei* Rut-C30 grown on Rice bran and corn straw with ratio of 5:5 gave the maximum production of Cellulase on the 6th day (18.5 IU/ml) [9]. Fagade and Bamigboye observed the 0.44 mg/ml cellulase production by *Bacillus subtillis* on day 4 at 40^{o}C [2] (Table 1) (Figure 4).

Figure 3: Multivalent enzyme producing Endophytic bacteria shows zone of various enzymatic activity.

Figure 4: Cellulase enzyme producing bacteria.

Conclusion

On basis of results obtained it can be concluded that three species of Bacillus (MSZLd, MSZLe and MSZPse) and Cocci (MSZLc) out

of these four endophytes isolated from leaf and one from seed part of plant, they have proved to be potential multivalent rich in various enzymes in addition to their plant growth promoting attributes under *in vitro* conditions. Further studies are, however, needed to investigate the mode of action of these strains in terms of inducing systemic resistance and enhancing their antibiosis activity against fungal pathogens, as well as confirming the antagonistic ability of these strains in field trials in lemon plant.

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Conflict of Interest

The authors have no conflicts of interest in preparing of this research article.

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