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International Journal of Current Research
Vol. 6, Issue, 03, pp.5346-5348, March, 2014

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

PRODUCTION OF BACTERIOCIN BY *Rhizobium* ISOLATED FROM RHIZOSPHERE SOIL OF MAIZE IN LALGUDI TALUK, TRICHY DISTRICT, TAMILNADU, INDIA

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ARTICLE INFO

Article History:
Received 10th December, 2013
Received in revised form
19th January, 2014
Accepted 17th February, 2014
Published online 25th March, 2014

Key words:

Rhizobium, YEMA medium,
Maize, Bacteriocin.

ABSTRACT

Rhizobium species were isolated from the rhizosphere soil of maize plants and studied for their ability to produce bacteriocins. *Rhizobium*, a nitrogen fixing bacteria can live in the rhizosphere soil of non-leguminous plants, exist freely and entraps atmospheric nitrogen and converts the unreactive nitrogen molecule to ammonia, a form that is readily utilize by plants. Rhizosphere soil from maize plants were collected from Lalgudi Taluk, Trichy District and identified based on their morphological and biochemical characters. The efficient strains were used for the production of bacteriocin.

INTRODUCTION

Bacteriocins are proteinaceous toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strains. They are typically considered to be narrow spectrum antibiotics. Bacteriocins were first discovered by A.Gratia. He called his first discovery, a colic in because it killed *Eschericia coli*. Bacteriocins are agents which are encoded in the genetic material carried by plasmids, with the purpose of killing or inhibiting closely related species or different strains of the same species. Strains of *Rhizobium* inhabiting a single soil type may differ in symbiotic properties and other characters. The successful establishment of highly infective and effective nitrogen fixing strains in soil is of practical importance for successful maize production. Bacteriocin produced by *Rhizobium* may be one of the antagonistic or competitive factors are responsible for dominance of particular strains in maize fields. Bacteriocin production is also discussed in relation to competition or antagonism among soil bacteria. As bacteriocins act as pivotal substance in specific antagonistic bacterial interaction, they can be potentially used to control bacterial plant disease by exerting their lethal effects on bacteria of the same or related groups. *Rhizobium*-non legume symbiosis is the most promising plant bacterium association so far known. Inoculated *Rhizobium* strains often fail to compete with indigenous rhizobia and do not increase nodulation (Hafeez *et al*., 2005). Thus the successful use of rhizobial inoculants requires the knowledge of factors affecting the effectiveness and competitive ability of the rhizobia. One of the major factors reported to be affecting competition among rhizobia are bacteriocins (Oresnik *et al*., 1999). Bacteriocins constitute a heterogenous group comprising protein complexes or peptides with antibiotic effect against closely related species and strains (Tagg *et al*., 1976). Root nodule bacteria have been shown to produce bacteriocins, which have been grouped as small, medium and large based on their size and diffusion characteristics. The bacteriocins are heat tolarent, chloroform soluble and are less than 2000kDa molecular weight, Medium is generally bactericidal, heat labile and retained by cellophane and large defective bacteriophages (Hirsch, 1979). The term bacteriocin is used here to describe any inhibitory agent which causes antagonism between closely related strains and which is neither self-propagating (i.e.bacteriophage) nor an antibiotic with activity against a wide range of microorganisms. Bacteriocins are antimicrobial peptides with narrow or broad host ranges produced by numerous bacteria.

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MATERIALS AND METHODS

Sample collection

The rhizosphere soil from maize plants were collected from Viduthalaipuram in Lalgudi taluk, Trichy District.

Isolation of *Rhizobium* from rhizosphere soil

Decimal dilutions were prepared by suspending 1g in 10ml of sterilized dis H_2O. For isolation of *Rhizobium* strains aliquot from 10^{-3}, 10^{-4}were used to inoculate on YEMA medium in petriplates. The cultures were incubated at 30^0C for 24 hrs.

Identification of *Rhizobium*

Colony and cell morphology

Rhizobium strains were identified by colony morphology and gram staining (Gragham and Parker, 1964; Mahana, 1981; Vincent, 1970).

Biochemical test

Production of indole was noticed in inoculated tryptophan broth after 7 days of incubation by adding Kovac's reagent. The reduction of methyl red and voges- prouskauer reaction examined in glucose phosphate broth by adding methyl red and **α-** naptholsolution with KOH respectively. Citrate utilization was observed by using Simmon's Citrate medium with bromothymol blue in basal medium. Liquefication of gelatin was tested in 10% gelatin agar medium (Pohlman, 1931). Hydrolysis of starch was examined on starch with nutrient agar& iodine solution. Production of hydrogen sulphide gas examined by method described by Zobell and Feltham (1934). Production of ammonia from urea was examined by Christensen urea agar with phenol red as an indicator. Fermentation of carbohydrates was tested by adding 10% Andrade's indicator in the basal medium containing peptone water and 2% sugar. The Gram's staining to 1970 (Gragham and Parker, 1964; Mahana, 1981; Vincent, 1970). Catalase activity was observed by stirring the culture in a drop of hydrogen peroxide (10% by w/v), while oxidase activity was tested according to Kovac's (1956).

CRYEMA test

A 2.5 ml of congored dye was mixed with a litre of YEMA medium to prepare CRYEMA medium. Bacterial colonies of the YEMA medium were streaked on the CRYEMA medium and the petridishes were incubated at 28 ± 2^0C for 5-7 days.

Glucose peptone agar test (GPA test)

Rhizobial colonies from YEMA medium were transferred to GPA medium in a petridish by replica plating and observed for white colour colonies.

Microscopic observation

Bacterial cells in the CRYEMA medium were stained with carbol fuschin and visualized under a compound microscope. This dye stains the β-polyhydroxy butyrate granules in the *Rhizobium*. The cells of those colonies having β-polyhydroxy butyrate granules were picked up to inoculate on Bacteriocin production media.

Mass cultivation of *Rhizobium*

500 ml of YEMA broth was prepared in a conical flask. The pure *Rhizobium* colonies from CRYEMA medium was inoculated into YEMA broth. The flask was incubated at 37^0 C for 1 week.

Bacteriocin assay (Tagg *et al*., 1976)

To assay bacteriocin production, 24 hours old culture of indicator strain grown in Tryptone yeast extract medium, by mixing 1ml was with approximately 25ml of soft Tryptone yeast extract agar. Single colonies of the strains to be tested for bacteriocin activity were stab inoculated into the soft agar within two hours after the agar solidified. Halos were visible as cleared zones surrounding the stab inoculated cultures. The plates were scored after 48 hours of stab inoculation (Oresnik *et al*., 1999).

Extraction and purification of bacteriocin protein

Purification of bacteriocin was carried out by using procedure of Yang *et al.* (1992) and cell free Supernatant was used to carry out protein extractions. Twenty percent chloroform was added to CFS in a separatory funnel. The aqueous phase was saturated with cold ammonium sulphate and was kept overnight at 4^oC. The precipitate was collected by centrifugation at 15,000g for 30 min. The solid pellet was dissolved in distilled water and dialyzed against distilled water at room temperature for 24 hours. The suspension obtained was designated as proteinaceous fraction or crude bacteriocin fraction.

RESULTS AND DISCUSSION

In the present study, Rhizobial strains were isolated from the rhizosphere soil of maize plants. *Rhizobium* found to be having circular colonies with regular borders, convex, whitish pink in colour and glistering. Under light microscope, the isolates were non motile and were Gram negative. They were non sporing forming and aerobic (Table 1). The bacterium showed positive for Voges Proskauer, Citrate utilization, catalase, oxidase, TSI, carbohydrate fermentation (maltose, galactose, arabinose) and negative for Indole production, Methyl red, Urease, gelatin hydrolysis and starch hydrolysis (Table 2). The efficient strains of *Rhizobium* were identified by inoculating on CRYEMA medium and GPA medium. On CRYEMA medium, Rhizobial cells form red colour colonies and on GPA, they forms white, circular, entire raised convex colonies.PHB granules were seen when the Rhizobial cells were stained with Carbol fuschin. The pure Rhizobial colonies were mass cultivated in YEMA broth.
The mass cultivated Rhizobial colonies were used for the production of bacteriocin and it was extracted and purified, mixed with the distilled water to obtain crude suspension. The antibiotic-like substances produced by *Rhizobium* and some cowpea rhizobia are termed bacteriocins using the broad definition of Reeves, primarily based on their restricted host range. Vincent, (1970) suggested as a substituted adenine nucleotide (molecular weight 1,100). They as and suggested classification of agrocin 84 as a new type of bacteriocin. The assay methods and choice of indicator largely determine the number of antagonists detected and the type of antagonism involved (Schwinghamer, 1971). Protein-containing bacteriocins were not detected in slow-growing rhizobial strains. Since representative strains of *Rhizobium japonicum* were tested under a variety of cultural and experimental

conditions. Roslycky's, (1967) suggest that macromolecular and smaller protein-containing bacteriocins are rarely produced by slow-growing rhizobia.

Table 1. Cultural & morphological characters of *Rhizobium* isolates

S.No	Characters	Observation
1.	Shape	Circular
2.	Color	Whitishpink and Glistering
3.	Elevation	Convex
4.	Margin	Regular
5.	Opacity	Opaque
6.	Motility	Non-motile
7.	Bacterium shape	Rod
8.	Oxygen demand	Aerobic
9.	Spore forming	Non-spore forming
10.	Gram's nature	Gram negative

Table 2. Biochemical Characters of *Rhizobium* isolates

S.No	Test	Results
1.	Indole production	Negative
2.	Methyl Red	Negative
3.	VogesProskauer	Positive
4.	Citrate utilization	Positive
5.	Catalase	Positive
6.	Oxidase	Positive
7.	TSI	Positive with H_2S Production
8.	Urease	Negative
9.	Carbohydrate fermentation(Maltose)	Positive
10.	Carbohydrate fermentation(Galactose)	Positive
11.	Carbohydrate fermentation(Arabinose)	Positive
12.	Gelatin Hydrolysis	Negative
13.	Starch Hydrolysis	Negative

Bacteriocin production appears to be a common property regardless of ability to nodulate or fix nitrogen. Quantitative production was not related to symbiotic properties of strains or with a particular serotype. However, bacteriocin production, along with other characteristics, was useful in subdivided *Rhizobium*. The importance and prevalence of these groups in nature are not known, but they exemplify genetic diversity within the species. The ability of soil bacteria to produce bacteriocins, defined as specific, nonself-propagating inhibitory agents causing antagonism between closely related strains, and bacteriocinogenic activity has been described in almost all rhizobial species. Thus, bacteriocins have most of the properties considered desirable for microbial control (Triplett and Sadowsky, 1992l; Gray *et al*., 2006; Riley and Wertz, 2002 a). Bacteriocins play a fundamental role in the ecology of microbial populations, but little is known about the comprehensive interactions at an ecological and evolutionary level in diverse populations (such as biofilms) (Chen and Hoover, 2003). Bacteriocins are highly specific antibacterial proteins produced by strains of bacteria active mainly against some other strains of same or related sp(Gaur *et al.,* 2004).

Bacteriocin research has been mostly focused on genetics, mode of action or on discovering novel bacteriocins. Recently the bacterial ecology and evolution of these antimicrobial peptides were examined in greater detail, slice little is known about their roles in bacterial communities.

Conclusion

The results of this study have shown that *Rhizobium* from rhizosphere soil of non leguminous plants are able to produce bacteriocin and it may play an important role in interspecific competitions. In this study the broad spectrum activity of *Rhizobium sp* from maize may help in the improvement of legume inoculants.

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