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Production of Bacteriocins by *Rhizobium* strains from *Sesbania sesban* (L.) Merr

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Twenty six *Rhizobium* strains isolated from root nodules of *Sesbania sesban* (L.) Merr. were studied for their ability to produce bacteriocins. Among them, only seven strains have the ability to produce bacteriocins after 48 hours of growth. The highest activity was shown by SSR-7. The bacteriocin of SSR-7 was optimized by cultural and nutritional conditions. The strain showed highest activity, when tryptone yeast extract medium was supplemented with glucose, galactose and peptone. The pH range for maximum bacteriocin activity was 6.0-7.0. The partially purified bacteriocin was found to be sensitive to proteases and insensitive to DNase and RNase. The activity remained at 80 °C for 5 min. SDS-PAGE analysis of bacteriocin showed the presence of 30 kDa protein.

Key words: Bacteriocin production, *Rhizobium* sp., *Sesbania sesban*, SDS-PAGE.

Bacteriocins constitute a heterogeneous group comprising protein complexes or peptides with an 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 (Hirsch, 1979).

The small bacteriocins are heat labile, chloroform soluble and are less than 2000 Da in molecular weight (Nirmala *et al*., 2001). *Rhizobium leguminosarum* bv. *viciae* strain LC-31 produced a medium typed bacteriocin, that was found to be highly effective against some strains of *Rhizobium leguminosarum* bv. *viciae* and *Agrobacterium* sp. (Hafeez *et al*., 2005). Bacteriocin activities decrease more or less sharply at the end of growth phase as a result of degradation by proteases (Hur *et al*., 2000). Bacteriocin production was also affected by the medium composition and cultural conditions, such as pH, temperature and agitation (Kim *et al*., 2006). Therefore, the optimization of environmental conditions is very important for the enhancement of bacteriocin production.

Sesbania sesban (L.) Merr. is an important green manure crop used to replenish the nitrogen content of soil. Very little is known about the bacteriocins of *Rhizobium* strains associated with *Sesbania sesban*. Hence the

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present work was taken up to study the bacteriocin producing ability of 26 *Rhizobium* strains isolated from root nodules of *S. sesban*.

MATERIAL AND METHODS

Identification of *Rhizobium* strains

Twenty six *Rhizobium* strains were isolated from root nodules of *Sesbania sesban*, collected from different regions of Andhra Pradesh, India. The identity of the strains as *Rhizobium* was confirmed by plant infection test (Vincent, 1970). A representative isolate from *S. sesban* was identified as *Rhizobium radiobacter* MTCC 8917 (=*Agrobacterium radiobacter*). Since *Agrobacterium* and *Rhizobium* are still treated as separate genera in *Bergey's Manual of Systematic Bacteriology* (Kuykendall *et al*., 2005), we used the term *Rhizobium* sp. with strain numbers as SSR-1 to 26.

Bacteriocin detection and assay

The bacteriocin producing ability of the strains was bioassayed by differed antagonism method (Mayr-Harting *et al*., 1972) and modified simultaneous antagonism method (Nirmala and Gaur, 2000). Bacteriocin activity was examined by adding 100 µl of sterile, filtered samples in to wells made on Tryptone-yeast extract (TY) medium (0.6 % W/V agar) seeded with log phase indicator strains (0.5 µl/100 ml of the medium). The producer strains were grown in TY broth for 5 d and cells were separated by centrifugation at 10000 x g for 20 min. The supernatant was filtered through 0.2 µm millipore filter and it was designated as cell free supernatant (CFS). The bacteriocin titre in arbitrary units/ml (AU/ml) is expressed as the reciprocal of the highest two-fold dilution showed a maximum zone of inhibition, multiplied by conversion factor. The diameter of each inhibition zone was measured.

Effect of pH, carbon and nitrogen sources on bacteriocin production

To test the effect of initial pH, the production media were adjusted to different pH values (pH 4.0-9.0) and inoculated with producer strain, and incubated for 48 h. After incubation, the activity was measured in AU/ml. To test the influence of carbon sources, each carbon source was added as 1% to TY medium. To study the effect of different nitrogen sources, 0.1% of each nitrogen source was added to the TY medium along with most effective carbon source.

Partial purification of bacteriocin

Partial purification of proteins was carried out using the procedure of Yang *et al*. (1992). Cell free supernatant (CFS) was used for protein extraction (Hafeez *et al*., 2005). 20% chloroform was added to the CFS in a separatory funnel. The aqueous phase was saturated with cold ammonium sulphate from 20-100% (w/v) saturations and gradually stirred with a glass stirrer for 15-20 min. The aqueous phase was kept overnight at 4 °C. The precipitate was collected by centrifugation at 15000 x g for 30 min. The solid pellet dissolved in distilled water and dialyzed against distilled water at room temperature for 24 h. The suspension obtained was designated as crude bacteriocin. All the different dialyzed material of 0.01g was added in 100 µl Tris HCl (pH 6.5) buffer and tested for inhibitory activity. The quantification of protein concentration was done by Bradford method (Thimmaiah, 1990). Bovine serum albumin (BSA) was used to construct the standard curve.

Enzyme and temperature sensitivity

The concentrated bacteriocin was treated with protease, RNase and DNase with a final concentration of 5, 10, 15, 20 and 25 µg/ml in 10 mM Tris HCl, pH 7.5 for 4 hours at 37 ^{o}C and residual activity was determined. Sensitivity to different temperatures (40, 50, 60, 70, 80 and 90 ^{o}C) was determined by incubating up to 30 minutes. After incubation, the samples were cooled and residual activity was determined (Nirmala *et al*., 2001).

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of 10% Sodium dodecyl sulphate (SDS) was performed (Laemmli, 1970). Electrophoresis was conducted at constant current of 40 mA for 10 h at 30°C. The gel was stained with coomassie brilliant blue.

RESULTS AND DISCUSSION

Among the 26 *Rhizobium* strains, 7 strains viz., SSR-2, 5, 7, 9, 11, 13 and 16 produced clear zones on tryptone yeast extract (TY) agar (0.6% w/v) medium against indicator strains of the same host used in this study (Table 1). The

bacteriocin production starts after 48 h and reached maximum after 96 h. Further incubation does not increase the zone size. Maximum activity (highest inhibition zone) was observed in the case of SSR-7 against SSR-10. This strain also showed the broad spectrum of activity, i.e., it inhibited maximum number of strains. The strain SSR-11 showed narrow spectrum of activity and it inhibited the growth of SSR-4 and SSR-26. Thus the activity spectrum varied from strain to strain was reported earlier in *Rhizobium leguminosarum* bv. *viciae* (Hafeez *et al*., 2005). As the strain SSR-7 showed highest inhibition zone, further studies were carried out for SSR-7.

Effect of pH on bacteriocin production revealed that the SSR-7 showed highest activity at pH range of 6.0-7.0. Above and below the neutral pH, activity decreases slowly (Fig. 1). In general, pH is known to be important for cell growth as well as the bacteriocin production because aggregation, adsorption of bacteriocin to the cells and proteolytic degradation depend on pH and can affect the bacteriocin activity in culture supernatant (Cheigh *et al.*, 2002).

The effect of carbon sources on growth and bacteriocin production was determined using TY medium supplemented with 1 % of different carbon sources. Relatively more growth was observed in media containing mannitol, glucose and galactose (Fig. 2). However, the highest bacteriocin activity was obtained with TY medium with galactose and glucose (148 AU/ml) and the activity was twice that obtained with mannitol (73 AU/ml). Thus the bacteriocin activity varied from one carbon source to other was also reported in *Micrococcus* sp. (Kim *et al.*, 2006).

Table 1. Screening of bacteriocin producing *Rhizobium* strains from *Sesbania sesban*

Producer strains	Indicator strains (diameter of inhibition zone in mm)								
	SSR-10	SSR-14	SSR-15	SSR-17	SSR-19	SSR-21	SSR-24	SSR-25	SSR-26
SSR-2	-	6.5	4.0	5.5	7.2	-	4.2	-	4.2
SSR-5	7.5	-	5.9	-	7.5	5.2	-	5.2	-
SSR-7	11.5	7.2	5.5	4.5	7.2	4.5	4.0	4.2	4.2
SSR-9	-	-	5.9	4.9	5.2	4.9	5.5	-	3.9
SSR-11	5.5	3.5	-	-	-	-	-	-	4.0
SSR-13	-	4.5	5.9	4.5	-	4.2	5.5	-	4.9
SSR-16	7.2	-	4.5	-	4.2	-	4.5	4.5	-

Table 2. Activity spectrum of efficient bacteriocin of strain SSR-7

Inbdicator strains	Potency	Diameter of inhibition zone (mm)*	Inhibition area (mm^2)*
SSR-7	-	-	-
SSR-4	++	7.2	40.6
SSR-10	+++	11.5	103.8
SSR-15	+	4.5	15.8
SSR-17	++	7.2	40.6
SSR-19	+	4.5	15.8
SSR-21	+	4.0	12.5
SSR-24	+	4.2	13.8
SSR-25	+	4.2	13.8
SSR-26	+	5.5	8.6

--: ineffective, +: less effective, ++: effective, +++: more effective.
* All the values are means of triplicates.

In order to assess the effect of the nitrogen source on growth and bacteriocin production, TY medium was supplemented with 0.1% additional nitrogen sources. Cell growth was greatly enhanced by the addition of peptone, beef extract, and soytone (Fig. 3). However, the highest activity was observed when the cells grown in the peptone containing medium (500 AU/ml), and bacteriocin activity twice that observed with the media containing other nitrogen sources. Therefore, TY medium with 1% galactose and 0.1% peptone were chosen as the optimum conditions for the maximum

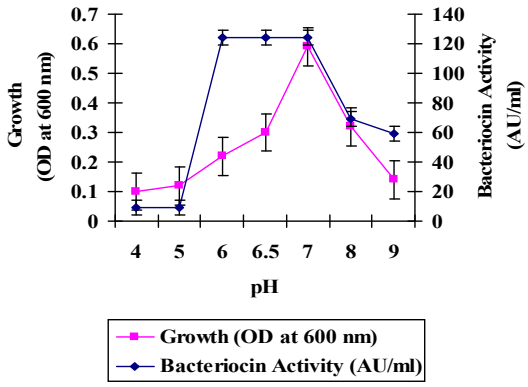

Fig. 1. Effect of pH on growth and bacteriocin production by *Rhizobium* strain SSR-7. Values are means of three independent determinations. Standard deviations (SD) are represented by error bars.

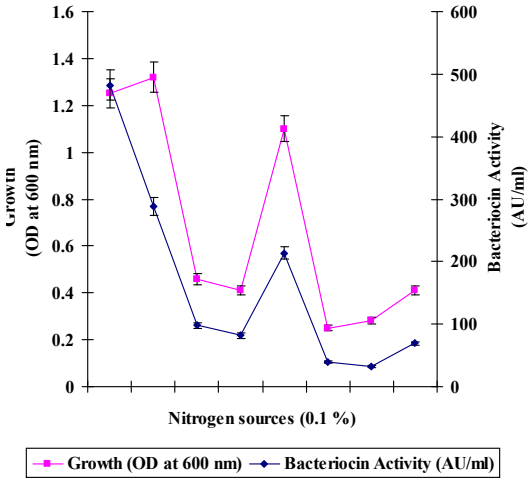

Fig. 3. Effect of nitrogen sources on growth and bacteriocin production by *Rhizobium* strain SSR-7. Values are means of three independent determinations. Standard deviations (SD) are represented by error bars.

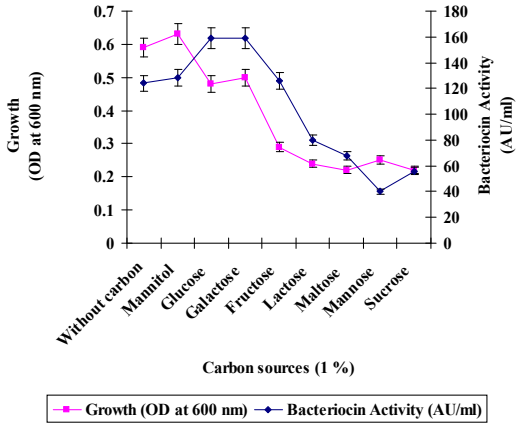

Fig. 2. Effect of carbon sources on growth and bacteriocin production by *Rhizobium* strain SSR-7. Values are means of three independent determinations. Standard deviations (SD) are represented by error bars.

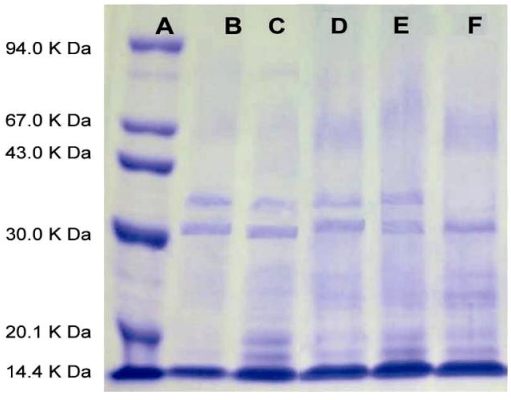

Fig. 4. SDS-PAGE analysis of partially purified bacteriocin by ammonium sulphate fractionation. A) Molecular weight marker, B) 30% ammonium sulphate, C) 40%, D) 50%, E) 60%, F) 70 %.

bacteriocin production.

In the purification steps of bacteriocins highest activity was observed in 70% ammonium sulphate saturated pellet and it was highly potent (Table 2). The protein concentration of bacteriocin at 70% ammonium sulphate saturation was low when compared to other fractions. This revealed that the activity does not depend on the quantity of protein produced, as reported earlier by Hafeez *et al*. (2005) in *R. leguminosarum* bv. *viciae*.

The purified sample was subjected to different temperatures (30, 40, 50, 80 and 90 °C) and the residual activity was determined. Optimum temperature range for bacteriocin activity was found to be 30-50°C, and above 50°C, activity decrease slowly. The activity remained at 80 °C for 5 min. At 90°C, the activity diminished. That the bacteriocin from *Cicer-Rhizobium* was found to be heat stable even after 5 min at 80°C was reported earlier by Nirmala *et al*. (2001).

The bacteriocin was sensitive to proteases at the concentration of 25 µg/ml indicating its proteinaceous nature. That the bacteriocins from *Cicer- Rhizobium* were sensitive to proteases was reported earlier (Nirmala *et al*., 2001). This bacteriocin unlike those of *R. leguminosarum* bv. *trifolii* (Schwinghamer, 1975) was insensitive to DNase and RNase indicating it is not a nucleoprotein. The bacteriocin was also found to be soluble in chloroform.

SDS-PAGE analysis of the protein isolated from SSR-7 showed the presence of 30 kDa protein band which is associated with inhibitory activity (Fig. 4). This band was visible only in samples isolated from 30-70% ammonium sulphate saturation of CFS. The molecular mass showed that this bacteriocin to be much smaller than those reported in *R. leguminosarum* bv. *trifolii* (Oresnik *et al*., 1983).

From this study it is clear that the cultural and nutritional conditions influence the maximum production of bacteriocins. Furthermore, the bacteriocin production may also play an important role in interspecific competition.

REFERENCES

1. Cheigh, C.I., H.J. Choi, H. Park, S.B. Kim, M.C. Kook, T.S. Kim, J.K. Hwang, Y.R. Pyun, Influence of growth conditions on the production of a nisin-like bacteriocin by *Lactococcus lactis* subsp. *lactis* A 164 isolated from Kimchi. *J. Biotechnol.* 2002; **95**: 225-235.
2. Hafeez, F.Y., F.I. Naeem, R. Naeem, A.H. Zaidi and K.A. Malik, Symbiotic effectiveness and bactericin production by *Rhizobium leguminosarum* bv. *viciae* isolated from agriculture soil in Faisalabad. *Environmental and Experimental Botany,* 2005; **54**: 142-147.
3. Hirsch, P.R., Plasmid-determined bacteriocin production *Rhizobium leguminosarum*. *Journal of General Microbiology*, 1979; **113**: 219-228.
4. Hur, J.W., H.H. Hyun, Y.R. Pyun, T.S. Kim, I.H. Yeo and H.D. Baik, Identification and characterization of lacticin BH5, a bacteriocin produced by *Lactococcus lactis*. BH5 isolated from Kimchi. *J. Food Prot*. 2000; **63**: 1707-1712.
5. Kim M.H., Y.J. Kong, H. Baek, and H.H. Hyun, Optimization of culture conditions and medium composition for the production of micrococcin GO5 by *Micrococcus* sp. GO5. *J. Biotechnol*. 2006; **121**: 54-61.
6. Kuykendall, L.D., J.M. Young, E. Martinez-Romero, A. Kerr and H. Sawada, Family I. *Rhizobiaceae*. Genus I. *Rhizobium*. In: Brenner DJ, Krieg NR, Staley JT (eds.), Bergey's Manual of Systematic Bacteriology, 2nd edn. Vol. 2, Springer Science, New York, USA 2005.
7. Laemmli, L.K, Cleavage of structural protein during the assembly of bacteriophage T4. Nature, 1970; **227**: 680-685.
8. Mayr-Harting, A., A.J. Hedges and R.C.W. Berkelaj, Methods for studying bacteriocins. In: Norris, J.R. and D.W. Ribbons (eds.), Methods in Microbiology, vol. 7A. Academic Press, New York. 1972; 315-422.
9. Nirmala, J. and Y.D. Gaur, Detection of bacteriocinogenic strains of *Cicer-Rhizobium* by modified simultaneous antagonism method. *Current Science*, 2000; **79**: 287-288.
10. Nirmala, J., Y.D. Gaur and P.K. Lawrence, Isolation and characterization of a bacteriocin by *Cicer-Rhizobium*. World *Journal of Microbiology and Biotechnology*, 2001; **17**: 795-799.
11. Oresnik, I.J., S. Twelker and M.F. Hynes, 1999. Cloning and characterization of a *Rhizobium leguminosarum* gene encoding the bacteriocin with similarities to RTX toxins. Applied and Environmental Microbiology, 65: 2833-2840.
12. Schwinghamer, E.A. 1975. Properties of some bacteriocins produced by *Rhizobium trifolii*. Journal of General Microbiol., 91: 403-413.
13. Tagg, J.R., A. Dajani and L.W. Wannanaker,

Bacteriocins of gram positive bacteria. *Bacteriological Reviews*, 1976; **40:** 722-756.

14. Thimmaiah, S.K., 1999: Estimation of soluble protein by dye-binding methods (Bradford's method). In: Standard Methods of Biochemical analysis, Kalyani Publishers, New Delhi, 97.
15. Vincent, J.M., A Manual for the Practical Study of Root Nodule Bacteria. IBP handbook15. Blackwell Scientific Publications, Oxford 1970.
16. Yang, R., M.C. Johanson and B. Ray, Novel method to extract large amount of bactericin from lactic acid bacteria. *Applied and Environmental Microbiology*, 1992; **58**: 3355-3359.