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NUTRITIONAL REQUIREMENTS AND INVERTASE ACTIVITY OF *RHIZOBIUM* NODULATING *SESBANIA SESBAN* ROOTS.

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Abstract

Carbon and nitrogen sources and pH range controling growth of *Rhizobium sesbaniae* localy isolated from root nodules of *S.sesban* (L) Merrill were studied in vitro. Some factors affecting extracellular invertase activity of the bacterium were investigated. The fast growing strain showed excellent growth on most of carbon sources tested, while it cannot grow on either starch or cellulose. The survey of nitrogen sources showed that uracil and phenyl alanine had a stimulatory effect, while ammonium chloride and adenine inhibited the growth of the bacterium. The minimum, optimum and maximum pH values affecting the growth of the isolate were 5, 8 and 10 respectively. The invertase activity of *R.sesbaniae* was optimum after six days of incubation, pH 7 and 15 g/l sucrose in growth culture.

Introduction:

Rhizobia are quite varied in their nutritional requirements and accordingly are devided into fast or slow-growing strains (Glenn & Dilworth, 1981; Stowers and Elkan, 1984; Ahmad and Smith, 1985). It has been reported that the fast growing rhizobia grow well on disaccharides as sole carbon sources (Martinez de Drets et al., 1974, Trinick, 1980; Glenn & Dilworth, 1981).

The nodule bacteria were able to utilize various organic and inorganic forms of nitrogen (El-Gamal, 1977;

Chakrabarti et al., 1981; Stowers and Elkan, 1984, Mohapatra and Gresshoff, 1984; Ahmad and Smith, 1985). Optimum pH for rhizobia was neutral or slightly alkaline Yadav & Vyas, 1971). The ability of rhizobia to grow at wide pH was also recorded (Yadav and Vyas, 1973; Okafor and Alexander, 1975; Pandhler and Kahlom, 1978). Sucrose was the major carbohydrate in legume nodules (Martinez-De Drets et al., 1974; Singh et al., 1980; Streeter, 1981). Since sucrose is the principal sugar translocated to nodules, its hydrolysis by invertase may be an initial step in making glucose and fractose available to bacteroids (Singh et al., 1980).

The goal of this investigation is to throw light upon nutritional requirements of Rhizobium nodulating roots of Sesbania sesban (L) Merrill and some factors affecting production of extracellular invertase activity of the bacterium.

Materials and Methods:

R.sesbaniae was isolated from the nodules of Sesbania sesban (L) Merrill naturally grown in Egypt by the method described by Vincent (1970). Cultures were checked for uniform colony morphology using a Tween 40 medium (Lim, 1978) and was subjected to test for purity (Somasegran and Hoben, (1985).

The growth of the rhizobium was studied in yeast extract mannitol (YEM) medium (Vincent, 1970). (YEM)-congared medium was used for counting the colonies by plate count method (Vincent, 1970).

Effect of carbon, nitrogen sources and pH values on growth. Medium used for carbon utilization consisted of (YEM) with the omitting mannitol. Carbon was added at a final concentration of 1% (W/V) from a filtersterilized or autoclaved 10% (W/V) carbohydrate solution.

Medium for nitrogen utilization was (YEM) without yeast extract and the addition of 0.5g/l nitrogen source.

To test the tolerance of the strain to pH values, the pH of (YEM) medium used was by adjusting the pH to 3,4,5,6,7,8,9 and 10 using buffer (Howieson et al., 1988).

Determination of growth rates: Liquid cultures were used using a 2% inoculum (A_{520} = 0.1, 1cm length path) and incubated in a rotary shaker at 28°C. The bacterium growth was determined in response to different C,N and pH. A sample of the culture 0.1 ml containing $1x10^8$ cells were spread on plates with the desired factor. The plates were incubated at 28°C for 2-5 days and compared with control plates containing (YEM). Each treatment consisted of five replications in randomized blocks.

Assay of invertase activity: The bacterium was grown in triplicate at 28°C in 50 ml sterilized medium (pH 6.8) containing (mmol) sucrose 29.2; K_2HPO_4, 2.9; $MgSO_4$, 0.8; $CaSO_4$, 0.7; Ca CO_3, 10; NaCl, 1.7 and g/l yeast extract, 1. (Singh et al., 1980). Invertase enzyme was determined by following reductimetrically the hydrolysis of sucrose as described by Martinez-De Drets et al. (1974) and Singh et al. (1980). The effect of sucrose concentration of the growth medium, pH, incubation time and agitation of the growth culture on the production of free invertase was studied. Glucose was assayed using the method of Nelson (1944). Protein was determined by the method of lowry et al. (1955).

Results and Discussion:

Carbon utilization: Rhizobium of S.sesban showed consistent carbon utilization pattern (Table 1). Nearly the isolate utilized the tested carbon sources with the exception

of cellulose and starch as good energy sources. This is in accordance with El Gamal (1977) who found that Rhizobium sesbaniae could utilize many carbon sources efficiently. However, limited growth response was observed on glucose, mannose and ribose. Intermediates of the tricarboxylic acid cycle as well as acetate were generally used for growth by this isolate, although acetate showed the least growth response. This is in contrary with the results of Stowers and Elkan (1984) for the growth cowpea rhizobia. The fast growing tested strain appeared to use the disaccharides such as sucrose, maltose and trehalose efficiently. This agrees with the previous findings of (Martinez-De Drets et al., 1974; Singh et al., 1980, Glenn and Dilworth, 1981; Stowers and ElKan, 1984). It is interesting to note that although traditionally mannitol is used as the energy source for many rhizobia, gluconate and glycerol each gave a better growth response. This is in accordance with (ElKan and Kwik, 1968; Stowers and ElKan, 1984) who found that gluconate and glycerol could act as energy sources more efficiently than mannitol. They claimed that gluconate supported growth very well by making it the most generally usable energy source for many rhizobia.

Nitrogen utilization: Survey of nitrogen sources (Table 1), showed that certain amino acid (phenyl alanine) can satisfy the nitrogen requirement of R.sesbaniae. Uracil was the source of nitrogen best used by this species. Adenine and ammonium chloride were used by the bacterium, yet they showed 80 and 75% less growth than with yeast extract (control). Moreover, potassium nitrate, urea and thymine showed similar utilization patterns to yeast extract. Generally our data showed that Rhizobium sesbaniae responded differently with the tested nitrogen sources.

Similar results were recorded by El Gamal (1977) with R.sesbaniae except that he observed inhibitory effect when using phenyl alanine and urea. Chakrabarti et al. (1981) found that half of 85 Rhizobium strains examined could grow on yeast extract while some of them were inhibited by yeast extract, NH_4-N and NO_3-N. On the other hand Stowers and ElKan (1984) and Mohabatra and Gressoff (1984) reported that yeast extract was the source of nitrogen best used by 25 strains of cowpea rhizobia and Parasponia strain ANU 289 respectively.

PH tolerance: (Table 1) showed that Rhizobium sesbaniae could tolerate a wide range of pH values (5-10) with maximum growth at pH value of 8.0. The pH values from 5.0-7.0 supported growth while pH value of 10.0 was not lethal for its growth. PH values less than 5.0 (4.0 and 3.0) did not support growth. This results were in full agreement with the results obtained by Pandhler and Kahlon (1978) who observed that Rhizobium of Pisum sativum fail to grow at pH 3.0 and growth was attained at pH (6.5-8.0) while pH's above 8.5 were not lethal. On the other hand some strains of cowpea group rhizobia could grow at pH value as low as 3.5 (Okafor and Alexander 1975). Different strains varied widely in their sensitivity to pH. The pH 3.5, 3.0-3.5 and 3.0-4.0 were lethal for Sesbania cannabina, Crotalaria juncea and Glycine max strains respectively. The optimum pH for Sesbania cannabina was 5.5, for Crotalaria juncea strains 4.0-7.0 and for Glycine max strains 4.0-7.0 (Yadav and Vias, 1971; 1971a; 1973). PH 5.5 was optimum for growth of R.meliloti, peas Rhizobium and R.leguminosarum (Lowendrof et al., 1981; Helemish, 1981 and Helemish and El-Gammal, 1987) respectively.

Invertase production by R. sesbaniae: Reducing sugars appeared in the the sucrose-containing medium during

the growth of the bacterium. This indicated the extracellular hydrolysis of sucrose Fig. 1(a) Shaked culture enhanced invertase activity more than unshaked ones. The production of this enzyme increased and reached maximum activity at six days incubation (23 μ mol glucose produced/ min/mg protein) compared to (18.4 at the first day incubation). The enzymatic activity was increased with increasing sucrose concentration in media Fig. 1(c). At sucrose concentration of 15 g/l the enzymatic production was optimum. The maximum enzyme activity was attained at pH 7.0 Fig. 1(b). Martinez et al. (1974) observed similar results with different Rhizobium spp. when sucrose was used as the sole carbon source. Also the invertase activity produced from Neurospora crassa (Marzluf 1973) and from R.japonicum isolated from Sesbania grandiflora (Singh et al., 1980) are similar to our results. These results support the view that an extracellular invertate was present in R.sesbaniae root nodules of S.sesban and confirmed previous results on the fast growing rhizobia. Since the content of glucose was closely following the spicific activity of invertase, this enzyme, therefore, seems to play an important role in the utilization of nodular sucrose translocated from the photosynthetic organs. The slow growing strains of R.lupini which do not produce an intra or extra cellular invertase, and thus fail to metabolise sucrose upon culture in liquid medium are dependent on plant invertase for the hydrolysis of sucrose (Robertson and Taylor, 1973).

The significans of these observations in the culture of the isolated Rhizobium in the laboratory, is their ecological adaptation to particular environments.

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Table 1: Effect of carbon, nitrogen sources and pH values on the total viable counts of Rhizobium sesbani (local strain) and percentage difference from control.

Carbon			Nitrogen		
different C (1%)	No. of viable cells/mL culture $\times 10^6$	% difference	different N (0.5%)	No. of viable cells/mL culture $\times 10^7$	% difference
Mannitol (control)	5.33	00.0	Yeast extract (control)	5.30	00.0
Glucose	4.60	-28.9	Ammonim-chloride	1.30	-75.5
Galactose	7.40	+16.9	Potassium-nitrate	5.36	+01.1
Mannose	3.40	-46.2	Urea	4.90	-07.5
Xylose	10.28	+62.4	Phenyl-alanine	6.85	+29.2
Ribose	5.11	-19.3	Adenine	1.04	-80.3
Arabinose	11.51	+81.7	Uracil	7.16	-35.0
Sucrose	11.28	+76.9	Thymine	4.05	-23.6
Maltose	8.41	+32.9			
Trehalose	7.43	+17.4			
Glycerol	12.75	+100.6			
Sod. acetate	1.23	-80.6			
Sod. Succinate	9.66	+52.6			
Sod. gluconate	13.66	+114.8			
Starch	-	-			
Cellulose	-	-			
L.S.D.	0.060		L.S.D.	0.005	

	pH values							
	3	4	5	6	7	8	9	10
pH	0	0	7.02	7.43	7.90	8.50	7.00	5.62

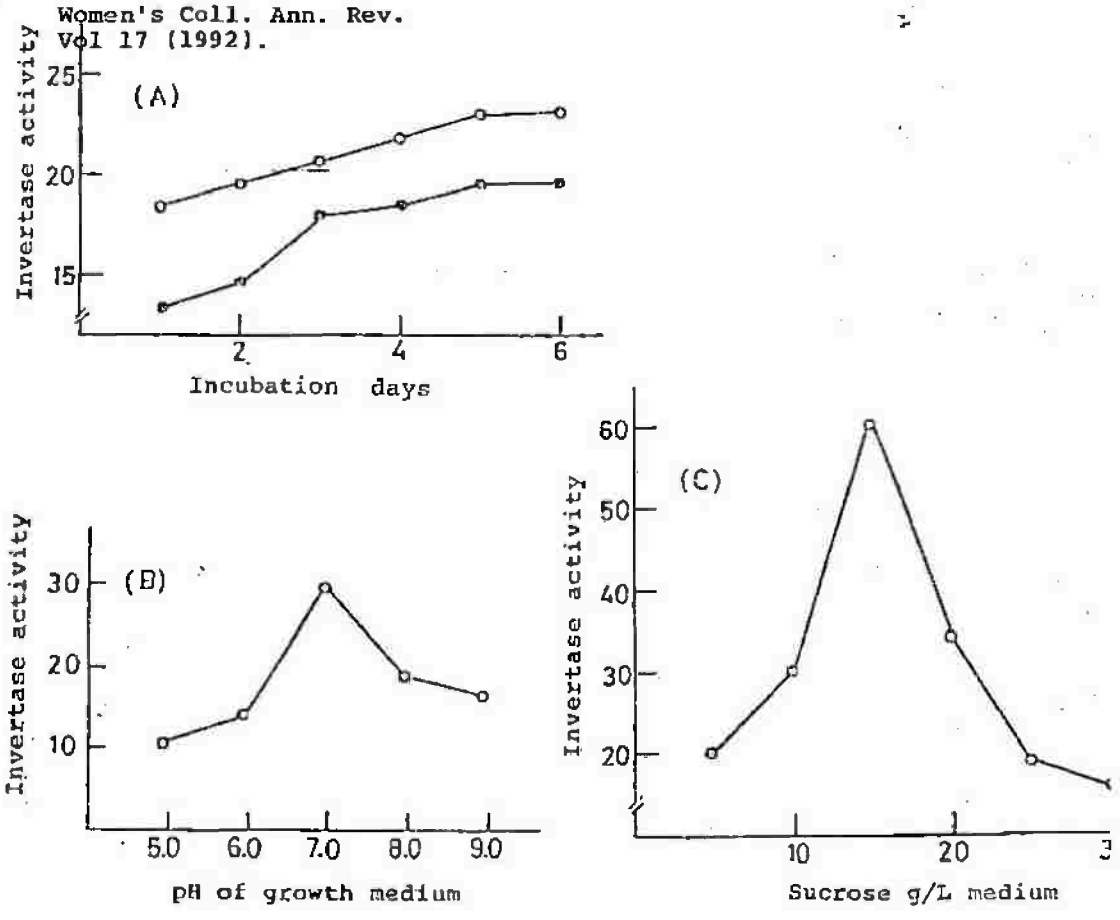

Fig. 1: Extracellular invertase activity produced by Rhizobium sesbaniae isolated from Sesbania sesban (L) Merrill as affected by:

(A) Incubation period of cells and shaking of the culture media

o——o shaked culture

●——● unshaked culture

(B) PH of the growth medium

(C) Sucrose concentration of the growth medium.

Invertase activity is expressed as µg glucose produced/ mg protein/ min.