Trans. Nat. Acad. Science & Tech. (Phils.)
1988: 10: 339-349

THE PRODUCTION OF GEL-FORMING POLYSACCHARIDES BY RHIZOBIUM SP. AND CURDLAN BY A MUTANT CULTURED IN COCONUT WATER

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ABSTRACT

The gel-forming polysaccharides (GFP) of selected tropical *Rhizobium* strains/isolates grown in coconut water (CW) were determined. After storage for six months at 4°C to 5°C, the strains were screened for curdlan (1→3, β-glucan) production. A mutant of *Rhizobium* spp. Niftal 600 formed blue colonies on glucose-yeast extract-aniline blue agar and coconut water-aniline blue agar medium. The other strains did not grow or exhibit blue colonies. The stability of the mutant to produce curdlan was determined by successive transfers twice a month for a year and found to be very stable. Production of curdlan used coconut water containing 2 g NaCl, 3 g $CaCO_3$ and 20 g sucrose per liter as culture medium. The GFP forms a resilient gel upon cooling of a heated preparation at 80°C and 98°C. A low-set gel was formed at 60°C to 80°C and a high-set gel above 90°C. Less syneresis of the gels occurred at ambient room temperatures, than at low temperatures of 4°C to 5°C in the refrigerator. The gels were stable from pH 3 to 10.

Introduction

Rhizobium, a genus of nitrogen fixing bacteria which forms a symbiotic relationship with legumes has been successfully cultured in coconut water (CW) (Mamaril *et al.,* 1985). Most tropical rhizobium strains produced copious polysaccharides when cultured in a rich medium such as CW. There are several types of polysaccharides produced by which extracellular polysaccharides (EPS), capsular polysaccharides (CPS) and lipopolysaccharides (LPS) are the major ones. Recently, a new type of polysaccharide, the gel-forming polysaccharides (GFP) (Dudman, 1984) was found to be produced by several rhizobium species. It is a neutral polysaccharide that can be extracted by boiling water or one M NaOH from the cells. This neutral polysaccharide forms firm gels in water as low as 0.2%.

It has also been shown that a strain of *R. trifolii* (J60) (Ghai *et al.,* 1981) and *Rhizobium* spp. TISTR 64W and TISTR 64B (Footrackul *et al.,* 1981) produce GFP of the curdlan type which are polysaccharides composed mainly of (1→3)-β-

glucosidic linkages. This kind of GFP produces a firm, resilient and heat-irreversible gel when heated in aqueous suspension (Harada *et al.*, 1968; Saito *et al.*, 1968). This polymer was first reported on a mutant strain (10C3K) (Harada *et al.*, 1966) of *Alcaligenes faecalis* var. myxogenes 10C3. Several strains of *Agrobacterium* (Hisamatsu *et al.*, 1977) were also found to produce curdlan as well as water soluble succinoglycans.

Curdlan has potential uses in the food industry as gelling agents with ability to form two types of gels as non-caloric materials for slimming, as edible films and fibers which are soluble in alkali but insoluble in water, as water-holding agents in sausages, hams and starchy jellies, as a binding agent in spaghetti noodles and hamburgers, as thickeners and stabilizers in salad dressings and spreads and as deodorant in boiled rice. It can also increase viscoelasticity of food preparations such as noodles and jellies. Other special uses include support for immobilizing enzymes and binding agents in tobacco products. Curdlan also possesses marked antitumor activity against certain allogeneic tumors, particularly Sarcoma-180 in mice (Harada, 1979).

The objectives of this study are to determine the production of gel-forming polysaccharides by several tropical *Rhizobium* strains cultured in coconut water and to screen for strains or mutants that will form curdlan, a non-toxic polymer.

Materials and Methods

The *Rhizobium* strains/isolates used in this study are listed in Table 1. They were maintained in yeast extract mannitol agar.

The strains/isolates were cultured in CW obtained from slightly mature coconuts (9-10 months old). They were incubated for four days at ambient room temperature under shaken condition. The cells were harvested by centrifugation, freeze-dried and then stored in the freezer.

Boiling water treatment

200 mg of lyophilized cells were treated with five mL of boiling distilled water and maintained for 10 minutes in the water bath (100°C). The cells were filtered out and the extracted GFP was allowed to gel at room temperature. The gel was dried and the GFP percentage determined.

Molar NaOH Treatment

200 mg of lyophilized cells were treated with five mL of M NaOH for 10 minutes in a boiling water bath. The M NaOH extracted GFP was allowed to gel, then dried, and GFP percentage determined.

Screening for curdlan producers

6-month old stock agar cultures of the rhizobium strains/isolates stored under refrigeration were screened fro curdlan production using the aniline blue method

Table 1. Selected tropical *Rhizobium* strains/isolates used in this study

Strain/Isolate	*Source*	*Host Plant*
CIAT 3714	Centro Internacional de Agricultural Tropical, Colombia	Butterfly pea (*Centrosema pubescens*)
C_{11}	Dept. of Soil Science, College of Agric. Univ. of the Phil. Los Banos (UPLB)	– do –
BC_P3	BIOTECH Culture Collection	– do –
L_5	Dept. of Soil Science, College of Agric., UPLB	Ipil-ipil (*Leucaena leucocephala*)
L_{15}	Dept. of Soil Science, College of Agric., UPLB	– do –
NIFTAL 600	Nitrogen Fixation in Tropical Agricultural Legumes, Hawaii	– do –
M_4	Dept. of Soil Science, College of Agric., UPLB	Mung bean (*Vigna radiata*)
M_5	– do –	– do –
S_{38}	– do –	Soybean (*Glycine max*)
CMBS	Millet Breeding Station, South India	Cowpea (*Vigna unguiculata*)
BMBS	– do –	Black gram
GMBS	– do –	Green gram

(Nakanishi *et al.*, 1974; Anemura *et al.*, 1977). The aniline blue medium contained 100 mL tap water, one g glucose, 0.5 g extract, five mg aniline blue, 1.5 g agar and was adjusted to pH 7.0. The strains/isolates were pre-cultured in a medium containing 0.5% NaCl, 1% glucose and 0.5% yeast extract at pH 7.0. The rhizobium culture was allowed to incubate for four days under shaken condition at ambient room temperature before transferring to the aniline blue agar medium. The strains producing curdlan will show up as blue colonies after five days incubation at 30°C.

Stability tests of mutant strains producing curdlan

The stability of the mutant strains was tested by successive transfers twice a month continuously for one year on aniline blue agar medium.

Production of GFP (Curdlan Type)

Two liters of coconut water containing three g $CaCO_3$, 20 g sucrose and two g NaCl per liter were placed in a 4-L Erlenmeyer flask and sterilized at 121°C for 15 minutes. The CW culture was inoculated with a pre-culture of the NIFTAL 600 mutant strain and incubated for 7-10 days under shaken condition. Samples were taken out to determine growth, pH, sugars and polysaccharides every 24 hours for 7-10 days. After polysaccharide production has remained stationary, two N NaOH solution was added to the culture broth to dissolve the polymer. The culture broth was allowed to shake for an additional 10 minutes and then the broth filtered. The filtrate was neutralized with two N HCl solution. The precipitate was collected by centrifugation and washed. The swelled polymer was then freeze dried. The scheme of propagation of the GFP (curdlan type) is given in Figure 1.

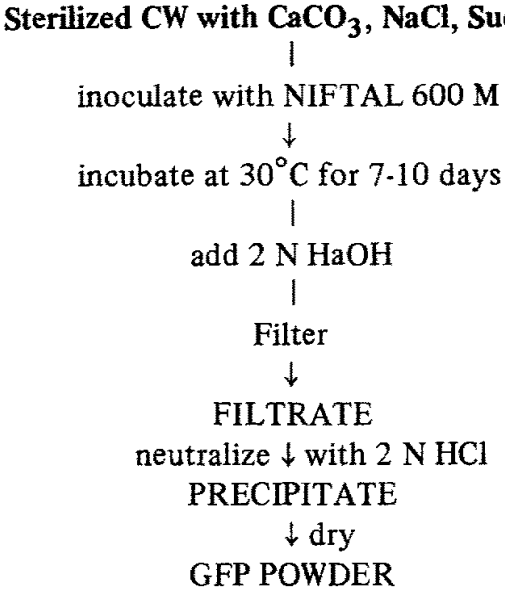

Figure 1. A schematic diagram for the production of NIFTAL 600 M gel forming polysaccharide powder.

Gel formation and gel strength of curdlan-type polysaccharides

A suspension of 1.5% GFP powder was heated and then allowed to cool at room temperature and the strength of the gel was then determined with a curdmeter. The same procedure was done on the same suspension heated at 55, 60, 70, 80, 90, 95 and 98°C.

Syneresis studies

A 1.5% suspension of GFP powder was heated to 97-98°C water bath for 10 minutes and allowed to cool until a gel is formed. One set was kept in the refrigerator and another set at ambient room temperature for two weeks. The percentage syneresis exudate was determined after four, nine and 14 days storage.

To study the effect of pH on the syneresis of the gel, a 1.5% polymer preparation was prepared by heating at 80°C and then the pH adjusted from pH 2.5 to pH 10 during the cooling period.

Results and Discussion

Rhizobium strains/isolates C_{11} and CMBS, GMBS, BMBS produced about 1-2% hot water-extractable GFP, BCIAT 3714 formed a colloidal polysaccharide solution, while L_5, L_{15}, and S_{38} produce the highest M NaOH-extractable GFP than the others. Theproduction of GFP by NaOH extraction tend to be higher than that of the hot-water treatment. BCIAT GFP formed colloidal solutions in both extractions. The results for seven selected isolates are tabulated in Table 2. One can note that the percentage of hot water and one M NaOH extractable GFP varied from one strain to another.

After six months of storage of the *Rhizobium* strains/isolates on agar slants in the refrigerator, the cultures were tested for production of GFP of the curdlan type. Only NIFTAL 600 developed a mixture of white and blue colonies. As shown in Figure 2, the colonies of the parent strain are different from that of the mutant NIFTAL 600 M. The mutant strain formed completely blue colonies while the parent strain formed white colonies with a bluish tinge in the center.

The stability of the mutant to produce curdlan was maintained even after more than 24 transfers. Completely dark blue colonies were produced in both glucose and CW-agar medium with aniline blue even after one year of successive transfers.

Table 2. The % extractable gel-forming polysaccharides of selected tropical rhizobium strains/isolates

Strain/Isolate	*% M NaOH extractable GFP*	*% Hot Water extractable GFP*	*% Basic soluble GFP*
L_{15}	6.71	–	6.71
S_{38}	5.46	–	5.46
BMBS	4.75	1.32	3.43
L_5	2.05	–	2.05
GMBS	2.82	1.99	0.83
CMBS	2.52	2.30	0.20
C_{11}	2.24	2.10	0.14

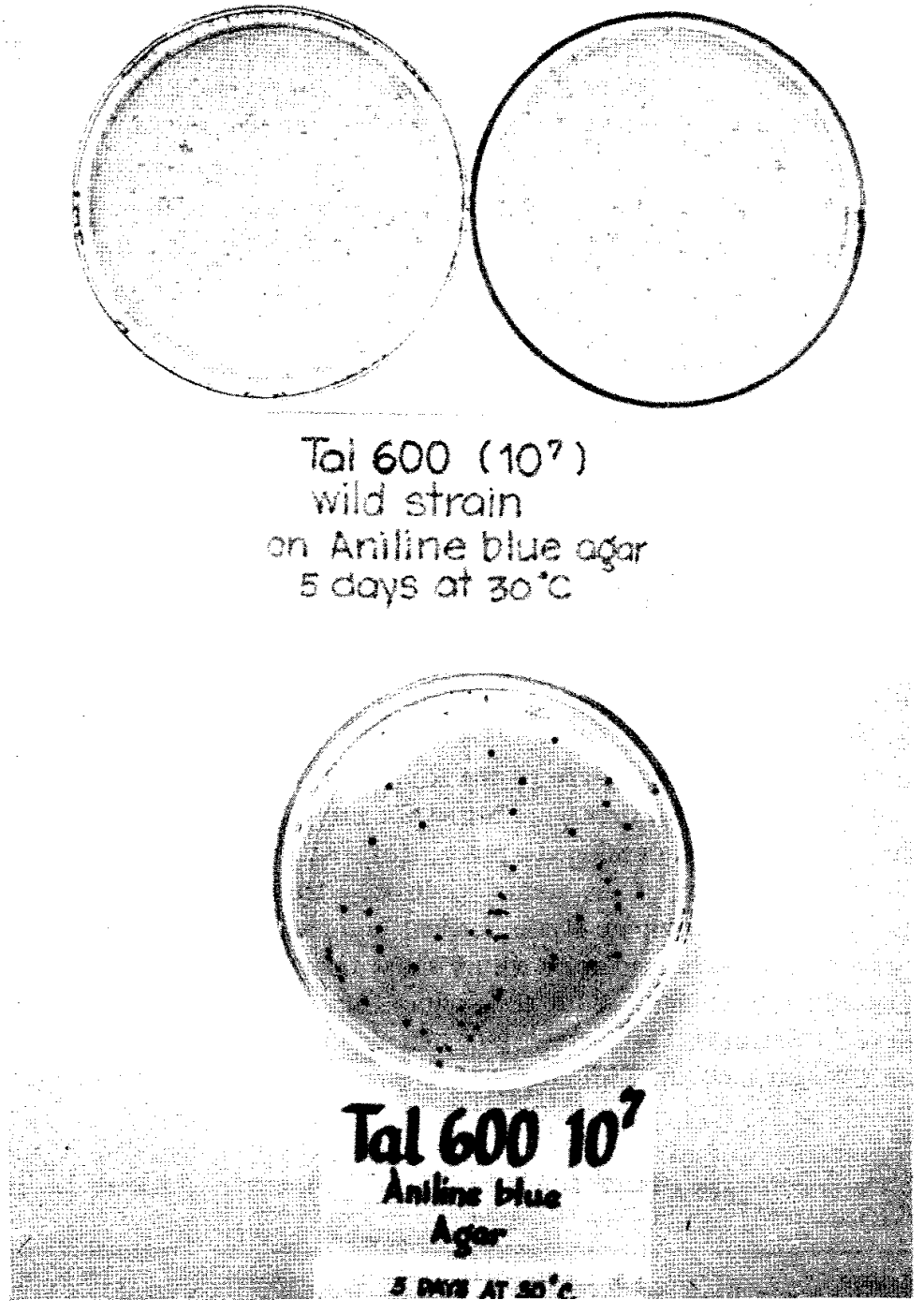

Figure 2. Photographs of colonies of the parent and mutant strain cultures in aniline blue agar medium.

(a) NIFTAL 600 wild type (parent strain).
(b) NIFTAL 600 M (mutant strain).

The production of GFP of the curdlan type from one liter of co3onut water following the scheme of preparation as shown in Figure 1 was about 2,000 mg GFP per liter. Conditions for optimum production of the polymer are being studied. An optimum image (40x) of NIFTAL 600 mutant colony is shown in Figure 3.

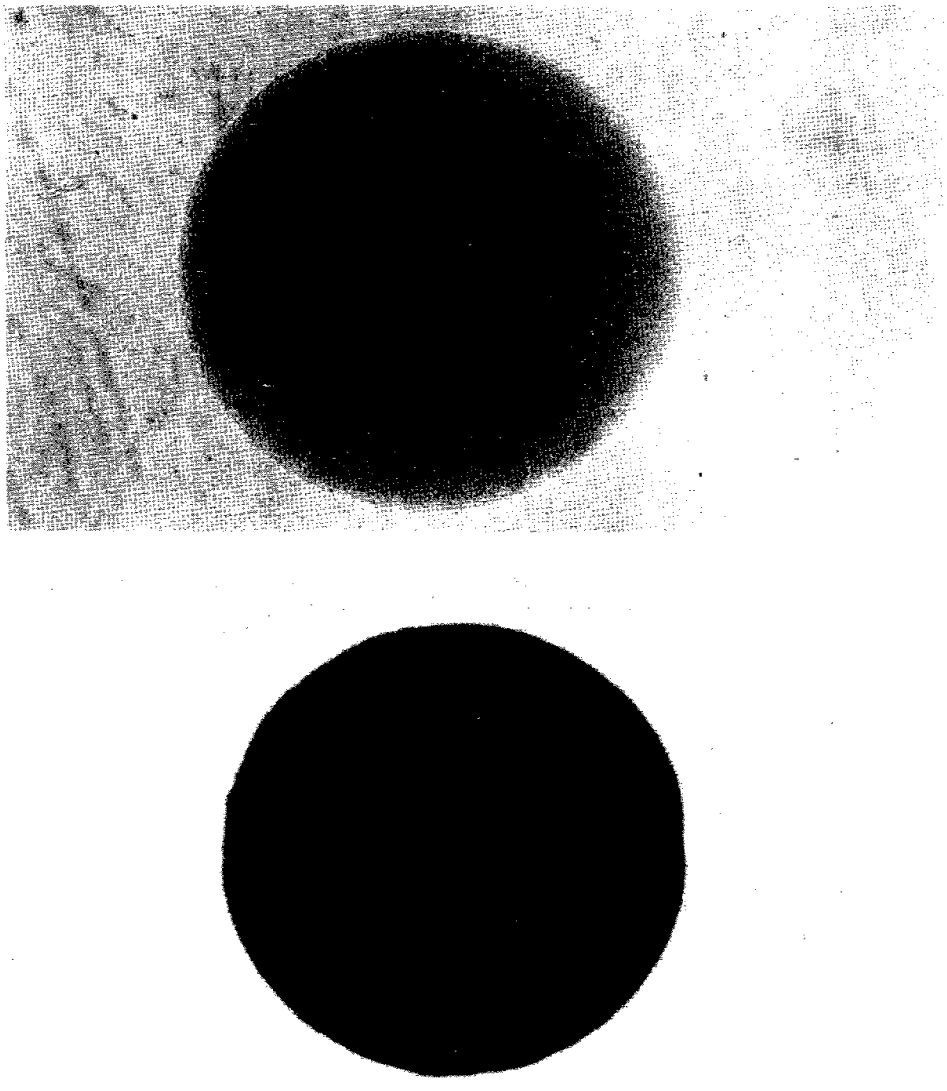

Figure 3. Photographs of a 5-day old colony of NIFTAL 600 (40x).

(a) Wild strain.
(b) Mutant strain.

Figure 4 shows a resilient firm white gel that was formed after heating a 1.5% suspension of the GFP (curdlan type) powder to 97-98°C for 10 min and cooling afterwards to room temperature.

The effect of heating temperature on the gel strength of NIFTAL 600 GFP gel is shown in Figure 5.

At temperatures below 60°C, the gel was too soft to determine gel strength by the curdmeter but as the temperature rose, gel strength increased. The gel strength between 60° and 80°C was gradual. After 80°C, the gel strength increased more rapidly with formation of a high-set gel.

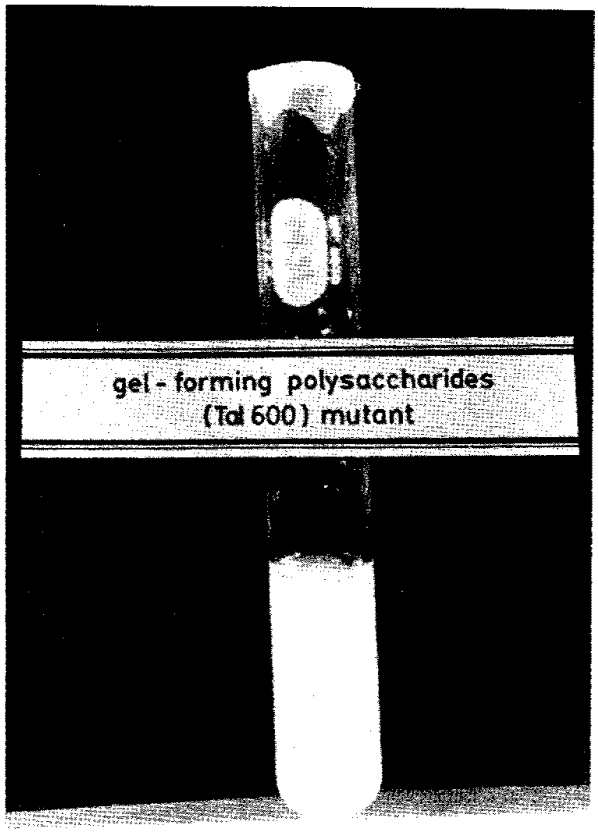

Figure 4. Photograph of a 1.5% aqueous suspension of gel forming polysaccharide (curdlan type) produced by *Rhizobium* sp. NIFTAL 600 M strain.

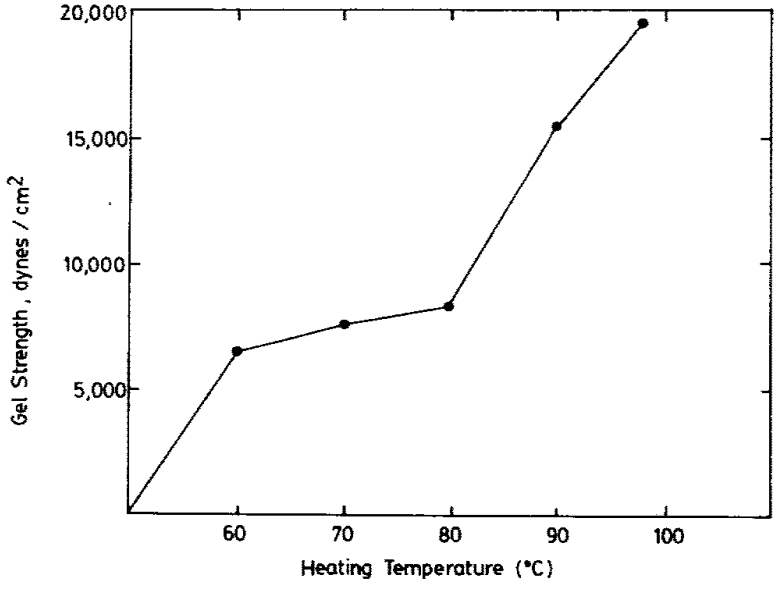

Figure 5. Gel strength of 1.5% gel-forming polysaccharide (curdlan) type) from *Rhizobium* sp. NIFTAL 600 M (Heating time, 10 min.).

Breakage of hydrogen bonds is required for gel formation in the first stages of heating. The firm gel formed during these stages after cooling may be due to the formation of hydrogen bonds (Harada, 1972).

The effect of storage temperaturn on the syneresis of NIFTAL 600 M high-set gel (Figure 6) showed much less syneresis at room temperatures than gels kept

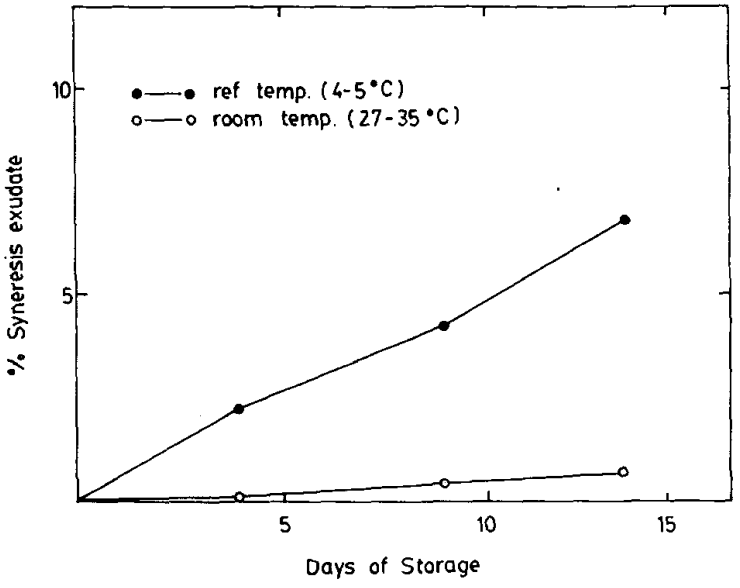

Figure 6. The effect of length of storage at refrigerator and room temperature on the syneresis of a 1.5% gel-forming polysaccharide of NIFTAL 600 M prepared by heating at 97-98°C.

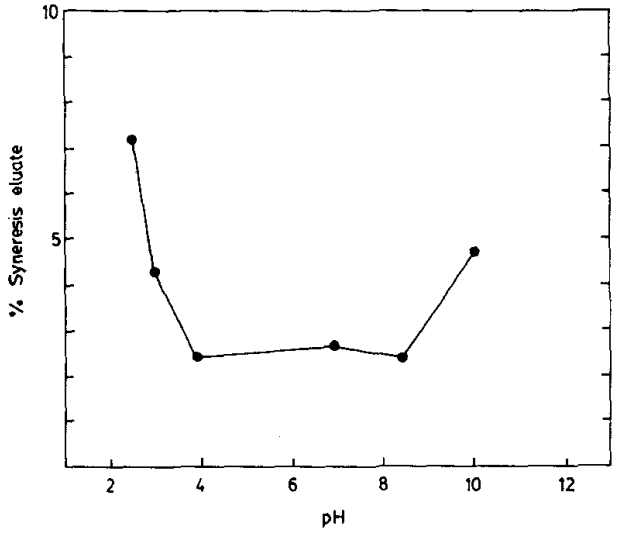

Figure 7. The effect of pH on syneresis of 1.5% NIFTAL 600 M gel prepared by heating at 80°C during storage for 4 days at 5°C.

in the refrigerator. This suggests that there is an increase of hydrogen bond formation at low temperatures which favor syneresis.

The effect of pH on syneresis of 1.5% gels prepared by heating at 80°C after four days storage at 5°C is shown in Figure 7. Syneresis was lowest between pH 4 to 8. The pH curve suggests that the gel is quite stable from pH 3 to 10 with syneresis exudate below 5%.

Summary and Conclusion

The gel-forming polysaccharides of several tropical *Rhizobium* strains/isolates were determined. CMBS with 2.33% and C_{11} with 2.10% gave the highest hot water-extractable GFP. While L_5, L_{15} and S_{38} showed only negligible amounts. On the other hand, L_{15} with 6.71% and S_{38} with 5.46% gave the highest M NaOH extractable GFP.

The agar slants of the strains after storage in the refrigerator (4-5°C) for six months were screened for GFP of the curdlan type (β-1→3 glucan). Only NIFTAL 600 showed a mixture strain was designated as NIFTAL 600 M. It formed completely blue colonies and produced substantial amounts of curdlan. The stability of the mutant for curdlan production was maintained in more than 24 transfers for a year.

The gel strength of NIFTAL 600 M GFP (curdlan type) increased with increase in heating temperature. Two kinds of gels were formed, a low set-gel (60-80°C) and a high-set gel (>90°C). Less syneresis occurred for gels stored at ambient room temperature than at lower temperatures in the refrigerator. Syneresis was a minimum between pH four to eight. Less than 5% syneresis exudate was observed for the gels between three to 10 suggesting that the gels were stable in this pH range.

Acknowledgment

This research work was supported by BIOTECH, UPLB. The authors wish to thank Ms. Lorele C. Trinidad, for assistance in the conduct of the study, Ms. Evelyn E. Carale for the preparation of the manuscript, and Prof. William Fernandez for his suggestions and encouragement.

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