Romanian Biotechnological Letters *Vol. 14, No. 4, 2009, pp. 4530-4537*

ORIGINAL PAPER

Curdlan-type polysaccharide obtained using a strain of *Agrobacterium rhizogenes*

Received for publication, March 13, 2009
Accepted, June 26, 2009

MIŞU MOSCOVICI[*], CORINA IONESCU, TUDORA CARAIANI, ANGELA CĂŞĂRICĂ, MIHAELA CLAUDIA MARINESCU, VASILICA ZĂHĂRĂCHESCU, DANIELA GHERA, ELEONORA GHEORGHIU, ALINA STAN, MARIANA SOARE, RAMONA MIHAELA MARDALE
National Institute for Chemical Pharmaceutical Research and Development, 112 Vitan Ave, 031299 Bucharest, Romania
[*] Corresponding author: mmisu@ncpri.ro, Tel: +40213212117

Abstract

*The strain nature-isolated of Agrobacterium rhizogenes **IBB-1601**, aerobic bacteria, was cultivated on a medium containing glucose as carbon source and corn steep liquor as nitrogen source, enriched with salts, with aeration and stirring conditions, at 28°C.*

Curdlan type polysaccharide was biosynthesized reaching a concentration of 30.7 g/l after 54h fermentation (productivity 0.56g/lh).

By processing of the fermentation broth a final product containing 70-88% polysaccharide was obtained.

Analytical characterization has revealed a structure of curdlan type.

Curdlan is a food additive and potential anti-tumoral agent.

Keywords: curdlan, biosynthesis, polysaccharide, *Agrobacterium rhizogenes*.

Introduction

Microbial polysaccharides have many practical applications in various industries as food, pharmaceutical, medical technology and cosmetics [1], and in the last decade new researches have been focused on the use of biopolymers (including curdlan) as vehicles of some bioactive substances to targeted cells and modulators [2].

Maximal final concentration of 64.4 g/l was obtained with an *Agrobacterium* collection strain, only on sucrose and mineral nitrogen source (diammonium phosphate), after 120 h (productivity 0.53 g/lh) during batch fermentation. The influence of agitation speed is considered to be important, because the dissolved oxygen level could become a limiting factor, but it seemed to be different from one strain to another. Because curdlan is water-insoluble, the fermentation broth presents a relatively low apparent viscosity during the bioprocess and therefore it is of low resistance to oxygen transfer, but the polysaccharide forms a surrounding resistant film around the cell, generally requiring a relatively high level of dissolved oxygen [16].

Curdlan, being a microbial biopolymer, belongs to the class known as (1,3)-ß-glucan macromolecules. These polysaccharides are characterized by repeating glucose subunits joined by a ß-linkage between the first and third carbon of the glucose rings [6-10].

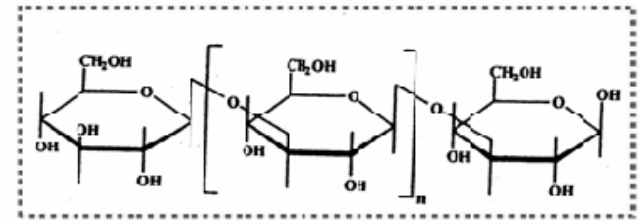

Figure 1. Structure of curdlan

In its natural state, curdlan is poorly crystalline and is found as a granule, much like that of starch. The granule is insoluble in distilled water, but dissolves easily in a dilute alkali solution, due to the ionization of hydrogen bonds, and forms a gel when it is heated above 54°C [8, 11].

The curdlan gel formation is influenced both by the differences in the concentration of sodium hydroxide used for dissolving it and by the temperature at which the gel is being prepared [11, 12].

In the current work, the curdlan - producing bacterial strain is a new one: *Agrobacterium rhizogenes* IBB-1601, which has been isolated from nature (of tumor plant roots).

Agrobacterium is a genus of gram negative bacteria, with the sizes 0,6-1,0 x 1,5-3,0 μm, single or in pairs, mobile because they use one or six peritrichous flagella; a non-sporogenic, aerobic microorganism which grows on media containing carbohydrates and releases large quantities of "mucus" [9, 10].

In this study we are presenting the fermentation dynamics with the new strain of nature-isolated Agrobacterium rhizogenes IBB-1601, as well as the biopolymer isolation and purification. Cultivated on glucose as carbon source and corn steep liquor as nitrogen source, its productivity reached the same level as was accomplished only with sucrose and ammonium phosphate by a collection strain [16].

Materials and Methods

Curdlan polysaccharide was obtained with a microorganism, *Agrobacterium rhizogenes* **IBB-1601,** from the collection of the Institute of Biology of the Roumanian Academy, Bucharest, which showed good results in lab experiences compared to other strains [14,15]. The colonies are smooth, convex, circular, unpigmented [16,17].

Preinoculum represents the support culture which was obtained during incubation at 28^{o}C (optimal temperature) in static conditions, on solid agarized medium, for 48-72 hours. It can be kept 30 days at 4°C. The medium composition (%w/v): glucose 1, yeast extract 0.3, malt extract 0.3, bactopeptone 0.5, agar 2.

The inoculum – biomass development on liquid medium, without product accumulation. It was prepared by drawing the preinoculum culture in 5 ml distilled water and using it to inoculate Erlenmeyer flasks of 500 mL with 100 mL liquid medium containing (%w/v): glucose 2; cornsteep liquor 1,5; KH_2PO_4 0,2; $MgSO_4$ *$7H_2O$ 0,05; the initial pH was 6,5: the medium was sterilized for 30 minutes at 115^{o}C. The cultures were incubated on a rotative shaker at 37^{o}C, for 48h. The final inoculum reached a growth level represented by an optical density of 9,4 (dil 1:5 at λ=570nm) and a final concentration of glucose of 0,1%.

Biosynthesis was performed on a liquid medium that contained glucose as main source of carbon and corn steep liquor as nitrogen source (%w/v): glucose 4; corn steep liquor 1; citric acid 0,2; KH_2PO_4 0,15; NaCl 0,2; $MgSO_4 * 7H_2O$ 0,05.

The fermentation was performed at 28°C with variable agitation conditions at 350-850 rpm (depending on the oxygen consumption) and aeration 0,5-0,7 v/vm. The biosynthesis experiments were carried out in a microfermenter Bio-Flo 2000 (New Brunswick Sci., USA) with a maximum available capacity of 10 l (working volume 7 l fermentation medium), and automatic control of parameters.

The fermenter has 200mm diam, 230 mm height (for 7 l broth), equipped with a Rushton impeller (6 flat blade disk turbine). The absorbance (DO) sensor had a response-time of 20-30 min.

Biomass separation

It was carried out by filtering on a Nutsche filter, under vacuum, with an adjuvant layer intermittently regenerated by scratching the fouled portion and washing the separated biomass; filtration was preceded by product solubilisation adding 10% (v/v) NaOH 5N solution and stirring for an hour.

Concentration and purification

To concentrate and purify the native solution (biomass free), ultrafiltration through semipermeable membranes was chosen, which can simultaneously ensure both concentration and purification, with reduced consumption of materials and energy, compared to classical techniques (concentration under vacuum and repeated precipitation, ionic exchange).

Thus, a concentration-diafiltration process by UF membranes was applied. It consisted in reducing the volume of solution by removing water and impurities, followed by the adding of a new volume of demineralized water or adequate solutions to the initial volume and a repeating ultrafiltration.

According to these procedures, a purified concentrate of biopolymer (macrosolute) could be obtained free of microsolute impurities, which permeate the membrane.

An ultrafiltration Pellicon module (Millipore), equipped with a cassette of plane polysulfone (PTGC) membranes having the separation limit of 10 kDa and a filtration area of 0.5 m^2, was used.

The alkalinity of the curdlan polysaccharide solution is high (0.5N) and may be harmful to the membranes, therefore an initial dilution of 1:22 (v/v) was applied.

Isolation of the purified polysaccharide

The usual isolation method for polysaccharides was applied: precipitation with an organic solvent water-miscible (usually an inferior alcohol) by adding, under vigorously stirring, of small amounts of the concentrated aqueous solution into ethanol at a ratio of 1:3 (v/v), followed by stirring it for 2 hours and maintaining it for 10 hours at 10°C.

Once the product decanted, it was re-suspended in alcohol, filtered and dried under vacuum, in temperature steps up to 85°C.

The bioprocess is shown in the scheme below.

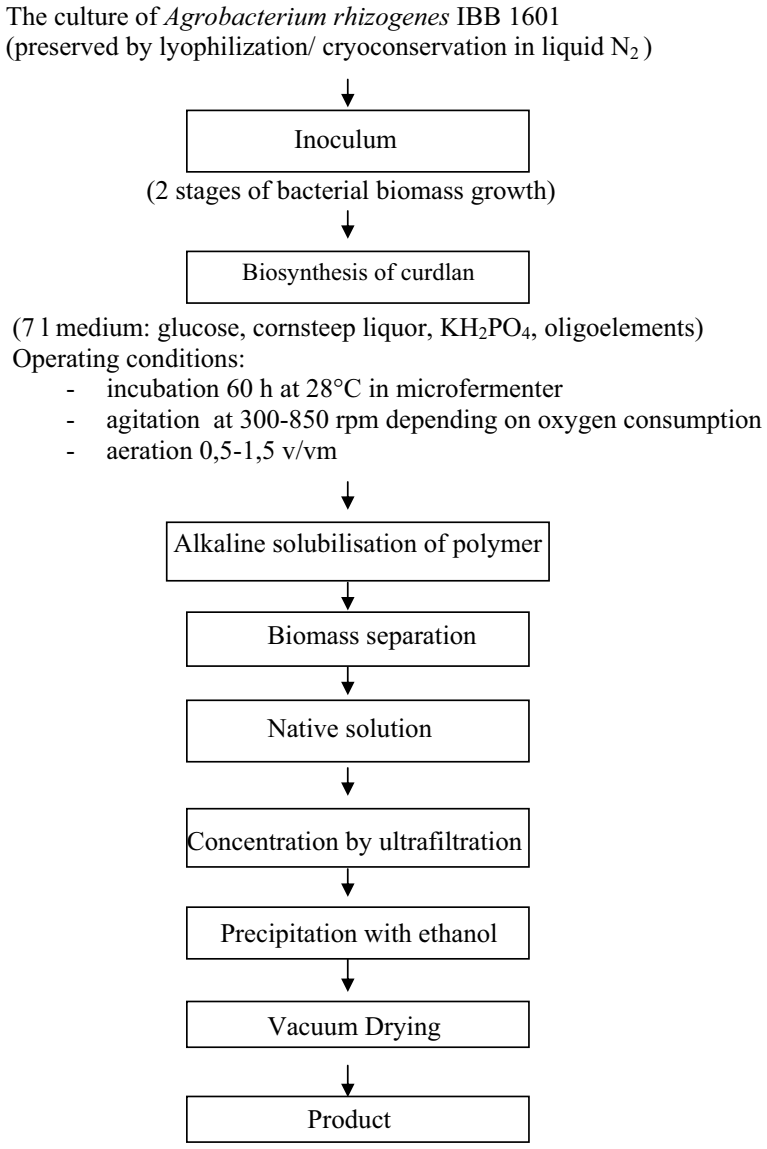

Fig. 2. Curdlan-type polysaccharide produced by *Agrobacterium rhizogenes* **IBB-1601**

Analytical methods: to assay the sugar concentration of the culture broth, the orto-toluidine method was used; biomass concentration was determined by measuring the optical density (D.O) (dil 1:50) by UV-VIS spectrophotometry (Helios γ, Thermo Electron Corporation) at λ = 570 nm, and the dry cell biomass, by dilution, centrifugation at 4000 rpm and drying the sediment at 105°C up to constant weight; the concentration of raw polysaccharide was determined by diluting the fermentation broth 1:1 (w/w), alkalisation with

NaOH 5N 1:10 (v/v), centrifugation at 4500 rpm, separation of biomass, product precipitation from supernatant with 3 volumes of ethanol, separation by centrifugation, washing with ethanol and drying the sediment under vacuum at 85°C until constant weight. The apparent viscosity of the fermentation broth was determined with a rotative Rheotest 2 viscometer (MLW, Germany) using the N cylinder, at shearing rates of 1300-600 s^{-1}.

Results and Discussion

Variation of pH, apparent viscosity, the level of dissolved oxygen and dynamics of a typical experimental curdlan producing fermentation, are presented in the figures 3-6.

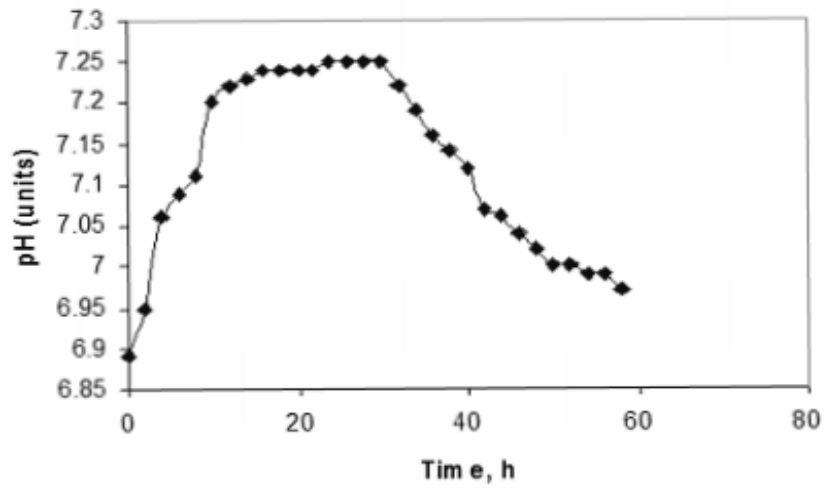

Fig. 3. Variation of pH during the fermentation with *Agrobacterium rhizogenes* **IBB - 1601**.

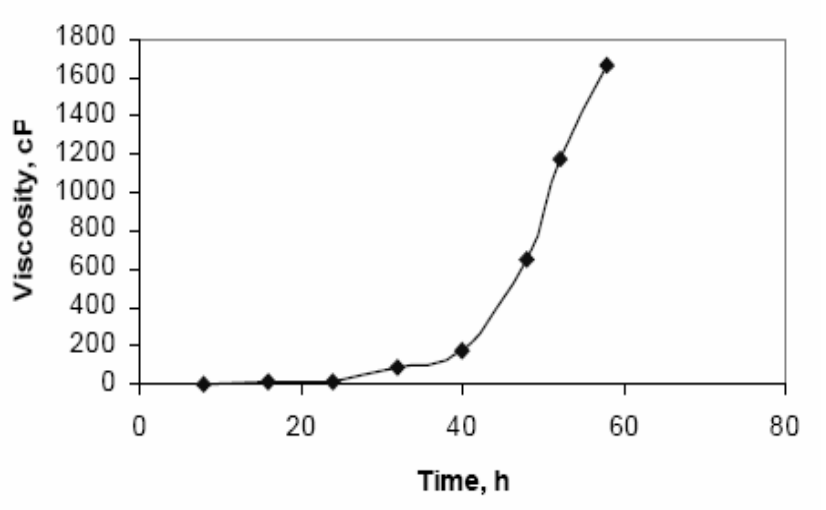

Fig. 4. The increase of the apparent viscosity of fermentation broth with *Agrobacterium rhizogenes* **IBB – 1601** during the bioprocess.

The sharp increase in viscosity during the period when the biosynthesis rate is already low suggests a polymerization which takes place in the extracellular environment, increasing the molecular weight of the biosynthesized polysaccharide.

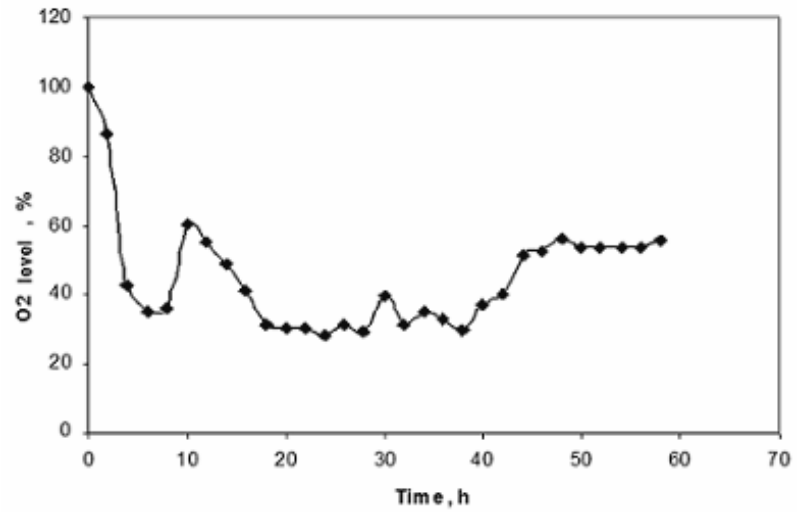

Fig. 5. The level of dissolved oxygen during the fermentation with *Agrobacterium rhizogenes* **IBB - 1601**

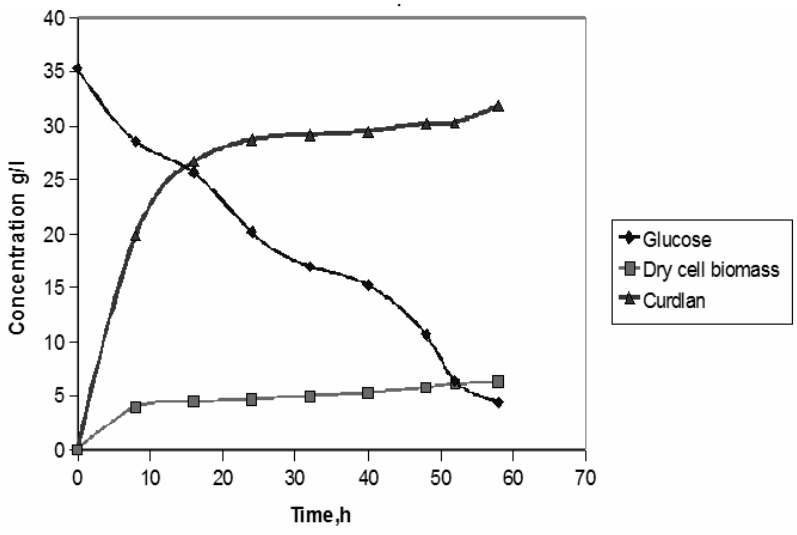

Fig. 6. Fermentation dynamics during production of curdlan polysaccharide by *Agrobacterium rhizogenes* **IBB-1601.**

The profile of the bioprocess characteristic curves showed that the biosynthesis was associated with cell growth.

Separation of biomass

In the following table are the quantitative results of biomass separation by fermentation broth filtration.

Table 1. Biomass separation by filtration of the fermentation broth (quantitative results)

Separation by filtration	Polysaccharide curdlan-type
Fermentation broth, l	7
Concentration, % (w/v)	3,2
Raw polysaccharide, g	224
Native solution, l	7,9
Concentration, % (w/v)	2,2
Raw polysaccharide, g	173,8
Yield, %	77,6

Isolation and purification of polysaccharide

The isolation and purification followed a generally, devoted and verified path of post-biosynthetic processing of extracellular polysaccharides broths: purification and concentration of native solution (cell free) and the isolation of polysaccharide by precipitation with water miscible organic solvent.

A special mention should be made for the isolation and purification of the curdlan polysaccharide, whose solubility only in alkaline solution of relatively high concentration (more than 0.5 N) and the tendency to form gel in aqueous solutions arose particularly difficult problems. Purification and precipitation yields were 30-50%, and 72-80% respectively.

Concentration and purification of native solution

The rejection coefficient (degree of retaining) of the biopolymer on the polysulfonic ultrafiltration membranes of 10kDa was determined using the relation:

$$\sigma = 1 - Cp/Cv$$

σ-rejection coefficient
Cp, Cv= the polymer concentration in permeate, respectively in the initial solution.

The semnificative value of rejection coefficient for the curdlan type biopolymer was 1.

Isolation of polysaccharide from the concentrated solution

Because of its exclusive solubility in concentrated alkaline solution, which interferes the obtaining of a high purity product (affected by the relatively high mineral content), demineralized water was used for washing the product after its precipitation and taking it up in ethanol, obtaining a product with a content of 82,4% (compared with the 80% set value for the commercial curdlan).

Conclusions

A strain of bacteria *Agrobacterium rhizogenes*, nature isolated **IBB-1601,** produced curdlan polysaccharide on a medium containing glucose as carbon source and corn steep liquor as nitrogen source, with a maximum final concentration of 31 g/l at 54 hours of fermentation (0.56 g/lh).

Taking into account the fact that the content of the raw product expressed in polymerized glucose was cca. 45 %, the conversion yield of the carbon substrate was 54 %. The pH presented a relatively constant value during biosynthesis, and the optimal level of dissolved oxygen was 30-40%**.**

The FT-IR spectrophotometric study (unpublished data) proved a curdlan-type biopolymer similar to the Takeda-Kirin commercial product, but also individualized due to the possible presence of a carboxyl group.

Acknowledgements

The works presented in this article were funded by the National Authority for Scientific Research -The National Center of Programs Management during the project CEEX 66/2006: "Bioshyntesis, chemical modification, analytical and immunological characterization of different polysaccharides micro and nanostructured, usable as adjuvants for a new generation of vaccines" (Imunobiopol).

References

1. LAROCHE C., MICHAUD P., *Recent Patents on Biotechnology,* 1, 59-73, 2007;
2. GUPTA R.K., SIBER G.R., *Vaccine* 13(4), 1263-1276, 1995;
3. KUN NA et al, *J. Controll. Release,* 69, 225-236, 2000;
4. KATAOKA K., *J. Biol. Chem.*, 277(39), 36825-36831, 2002;
5. DIWAN M., KHAR R. K., TALWAR G.P., *Vaccine*, 19(28-29), 3853-3859, 2001;
6. PHILIPS G. O., WILLIAMS P. A. eds., *Handbook of hydrocolloids,* CRC Press, Boca Raton, 2000, pp 269-286.
7. CHEESE MAN I. M., BROWN R.M. JR., "Microscopy of curdlan structure" *Howard Hughes Molecular Biology Summer Research Program,* Austin, TX, August, 1995;
8. HARADA T., MISAKI A., SAITO H., *Arch. Biochem.,* 24, 292-298, 1968;
9. MCINTOSH M., B. A. STONE, V. A. STANISICH, *Appl. Microbiol. Biotechnol.* 68, 163-173, 2005;
10. JEZEGUEL V., *Cereal Foods World,* 43(5), 361-364; 1998;
11. HINO T., ISCHIMOTO H., SHIMBAYA SHI S., *J. Pharm. Pharmacol.,* 4(55), 435-439, 2003;
12. NOBE M., KURODA N., DOBASHI T., YAMAMOTO T., KONNO A., NAKATA M., *Biomolecules,* 6(6), 3373-3379, 2005;
13. PHILLIPS K. R., LAWFORD H. G., *Prog. Ind. Microbiol.,* 18, 201-229, 1983;
14. ORTS W. J., ROUSSEAU J. D., LAWFORD H. G., *Industrial Polysaccharides*, Eds. S. Stivala, V. Crescenzi, I. C. M. Dea, Gordon Breach Science, New York, 1987, pp 459-465.
15. SAUDAGAR P. S., SHINGHAL R. S., *Appl. Biochem. Biotechnol.,* 118(1-3), 21-31, 2004;
16. LEE I. Y., M. K. KIM, J. H. LEE, W. T. LEO, J. K. JUNG, M. W. LEE, Y. H. PARK, *Bioproc. Eng.* 20, 283-287, 1999;
17. LEE J. H., M. K. LEE, M. K. KIM, Y. H. PARK *J. Ind. Microbiol.&Biotechnol.* 23, 143-148, 1999.