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Enzymatic synthesis of levan polysaccharide by *Bacillus licheniformis* levansucrase

Imen Dahech*, Rania Bredai, Karima Srih
Biochemistry laboratory, Faculty of sciences of Sfax, PB 802, 3018 Sfax, (TUNISIA)
E-mail : imenbiologie@yahoo.fr

ABSTRACT

Levan can be synthesized by the microorganism *Bacillus licheniformis*. The enzymatic synthesis of levan was carried out in erlenmeyers flasks and the influence of synthesis conditions was studied. The results show that the enzyme can produce an important amount of levan up to 80 g/l of levan when a concentration of 300 g/l of sucrose was used at pH 8 at 50°C for a period of 18h. The obtained results are also important making levan different from the previously reported levans and can be successfully used in industrial applications.

KEYWORDS

Bacillus licheniformis;
Levan;
Enzymatic synthesis;
Levansucrase.

INTRODUCTION

Levan, a polymer of fructose linked by fructofurano side bonds, is produced by the transfructosylation reaction of levansurcrase (E.C. 2.4.1. 10). Its molecular weight can reach values around 10^7 Da, corresponding approximately to 60,000 units of fructose[1]. Levansucrase is produced from various microorganisms and plants[2]. The sucrose hydrolysis by levansucrase can produce glucose, fructose, fructooligosaccharides and levan, however the concentration of each product depends on the initial sucrose concentration and on sucrose hydrolyze rate or fructose accumulation[3]. Recently, more interest has developed in the commercial production of levan, which offers a variety of industrial applications in the fields of cosmetics, foods and pharmaceuticals agent as a hypocholesterolemic and as an antitumor agent [4,5]. Levan presents applications as a thickening agent and a stabilizer in the food industry[6], besides its activity against tumor cells through modification of their membranes, increasing its permeability to cytotoxic agents and consequently facilitating these agents' action[7]. Other levan and fructose oligomer applications derive from its capacity to act as fibers, bringing good physiological and biochemical effects[8]. The rheological characterization of the levan is indispensable for its industrial applicability especially in the food industry. Several authors have critically emphasized the importance of biochemical and medium optimization strategies for levan production[9]. The main purpose of this work was to study the capacity of *Bacillus licheniformis* strain to synthesize levan after optimizing the synthesis conditions on levan production.

MATERIALS AND METHODS

Organism and culture conditions

The *B. licheniformis* strain was grown in a culture medium containing in g/l: Na_2HPO_4 3.5, NaH_2PO_4 0.8, $MgSO_4$ 0.2, $NaNO_3$ 3.5, yeast extract 5.0 and su-

crose 5.0. The incubation was carried out in a rotary shaker (200 rpm) at 40°C for 18h.

Fermentation conditions

The fermentation medium consisted in g/l: Na_2HPO_4 3.5, NaH_2PO_4 0.8, $MgSO_4$ 0.2, yeast extract 5.0 and sucrose 200. Following heat sterilization (121°C) for 20 min, the flask was inoculated with 5% (v/v) of the subculture and incubated for 24h at 40°C on a rotary shaker (200 rpm)[10].

Analytical methods

Enzyme extraction

After suitable fermentation time, the culture was centrifuged at 10,000 ×g for 15min to remove cells and the clear supernatant obtained was used for levan synthesis.

Reducing sugars

0.5 ml of enzyme extract was added to 0.5 ml of 20% sucrose (w/v) in 20 mM acetate buffer, pH 5.6, and the reaction mixture was incubated at 40°C for suitable time. The reaction was stopped and reducing sugars were determined by the DNS method[11].

Levan determination

0.5 ml of enzyme extract was added to 1.5 ml of 20% sucrose (w/v) in 20 mM acetate buffer, pH 5.6, and the reaction mixture was incubated at 40°C for suitable time. The levan was quantified in the supernatant by precipitation with ethanol 70% and determined according to Dahech et al.[12].

Effect of reaction time on levan synthesis

To study the effect of time reaction on levan synthesis, a 2 ml reaction mixture containing 10% of sucrose (w/v) in 20 mM acetate buffer (pH 5.6) and the crude enzyme were incubated for different incubation times and kept at 40°C. Reducing sugars and the amounts of produced levan were determined.

Effect of sucrose concentration on levan synthesis

To study the effect of sucrose concentration on levan synthesis, a 2 ml reaction mixture containing different concentrations of sucrose (10, 20, 30 and 40%) (w/v) in 20 mM acetate buffer (pH 5.6) and the crude enzyme were incubated for 18h and kept at 40°C. The amounts of produced levan were determined.

Optimum pH for levan synthesis

The optimum pH for levan synthesis was studied over a pH range of 3.0-10 at 40°C. The following buffer systems were used at 20 mM: sodium acetate (pH 3.0-5.0); phosphate (pH 6.0-7.0); Tris (pH 8.0); and glycine (pH 9.0-10) and the crude enzyme were incubated for 18h and kept at 40°C. The amounts of produced levan were determined.

Optimum temperature for levan synthesis

To determine the optimum temperature for levan synthesis, a 2 ml reaction mixture containing different concentrations of 10% of sucrose (w/v) in 20 mM acetate buffer (pH 5.6) and the crude enzyme were incubated for 18h and kept at various temperatures (40, 50, 60, 70 and 80°C). The amounts of produced levan were determined.

RESULTS AND DISCUSSION

Effect of reaction time

The time course data on levan synthesis by *Bacillus licheniformis* is shown in (Figure1). Levan was produced during exponential phase. Presented levansucrase can synthesize fructooligosaccharides (FOS) from the first hours and it is able to catalyze both the transfructosylation reaction (levan formation) and sucrose hydrolysis (liberation of fructose and glucose). In

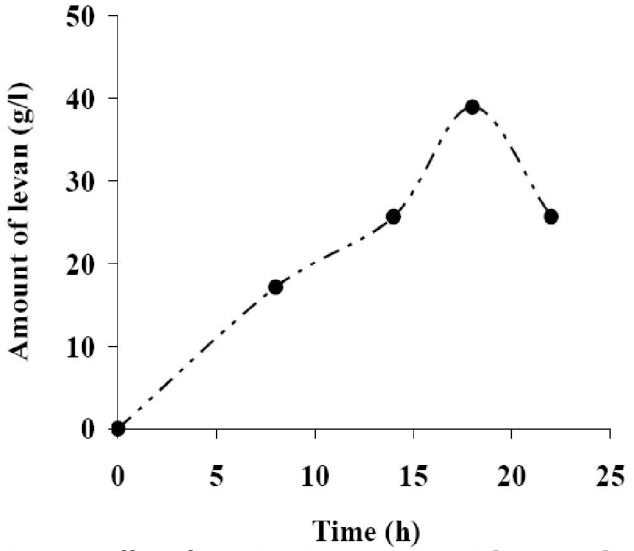

Figure 1 : Effect of reaction time on enzymatic levan synthesis by *B. licheniformis* strain

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fact, levan production increases with the time of synthesis. The optimum amount of levan was obtained at 18h to reach 40 g/l. Unfortunately, the levan production occurs when the initial concentration of sucrose in the medium is high, remaining high concentrations of reducing sugars (Figure 2). If the reaction is carried out for 20h, the amount of reducing sugars decreased and levan concentration decreased. Takahashi strain is an efficient levan-producing strain since it produced the highest yield of levan after 21h[13]. Sutherland (1999)[14] reported that several parameters influence the levan synthesis and the biopolymer yield may reduce or increase.

Effect of sucrose concentration

The polymer production remained constant (20 g/l) at lower sucrose content (10%). Further increase in sucrose concentration above 200 g/l strongly affected levan and the optimum (80 g/l) was obtained with 30% of sucrose (Figure 3). Above the concentration of 300 g/l of sucrose, the levan synthesis was noted to decrease.

According to Park and Baratti (1993)[15], many microorganisms bear high sugar concentrations for levan production. Vinhas (1999)[16] obtained maximum formation of levan by *Z. mobilis* strains with sucrose concentration of 200 g/l. Ahmed et al. (2005)[17] stated that sugar concentrations above the 20% may create problems due to the high viscosity caused by the polymer. Moreover, Hettwer et al. (1995)[18] reported that at higher sucrose concentrations, levan production at the beginning is high but later, its synthesis is inhibited. Then the hydrolase activity increases, accumulating oligosaccharides and increasing glucose concentration that continues inhibition of levansucrase.

Optimum temperature for levan synthesis

The levan formation during incubation at different temperatures (40-80°C) was studied and the results are shown in Figure 4. Maximum amount of levan (142 g/l) was produced at 50°C with initial pH 5.6, while low amount of levan (120 g/l) was produced at 40°C. These results suggest that at higher temperature (up to 40°C) and likewise at higher sucrose concentration, the transfructosylation reaction was preferentially performed. These results are also important characteristics of this levansucrase making it different from the previously reported levansucrases. Dolle et al.[19] verified that 25ºC at pH 5.0 is the ideal condition for levan production. Moreover, Reiss & Hartmeier[20] demonstrated good productivity of this polymer at temperature vary-

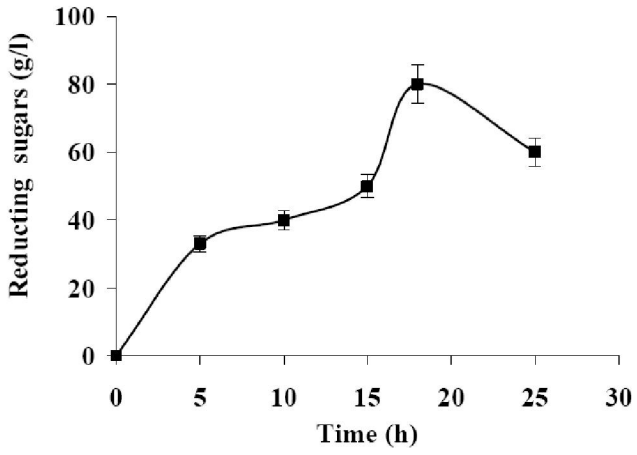

Figure 2 : Production of reducing sugars during levan synthesis

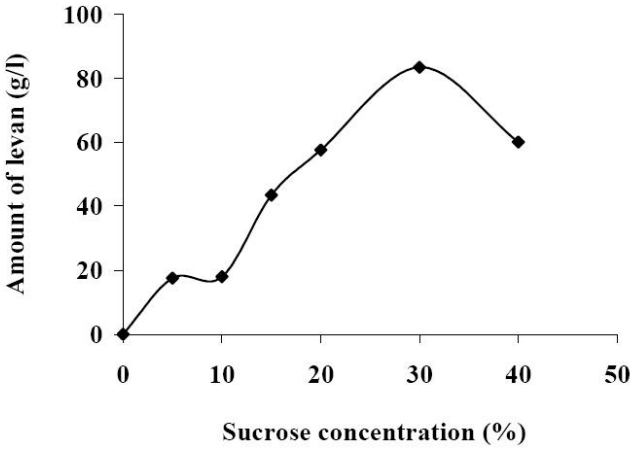

Figure 3 : Effect of sucrose concentration on levan synthesis

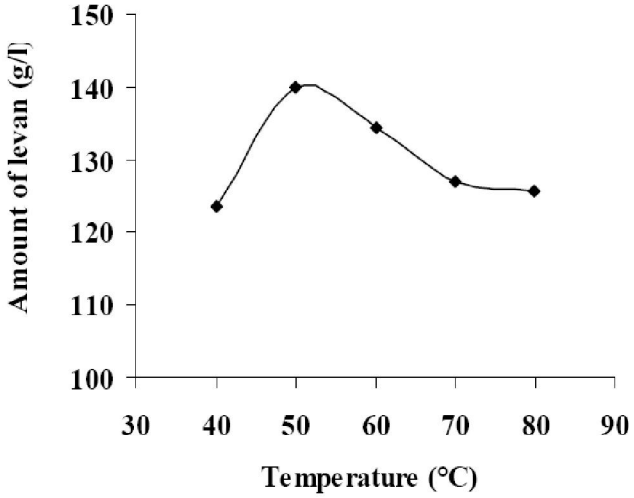

Figure 4 : Effect of temperature on levan synthesis

ing between 27-30ºC at pH 5.0.

Optimum pH for levan synthesis

A maximum concentration of levan 70 g/l was produced from the reaction at pH 8 with the sucrose concentration of 300 g/l at 40°C (Figure 5). Less levan production occurred at acid pH. The results indicated that the optimum pH for levan production lies within 7 and 8. Reiss and Hartmeier[20] demonstrated good productivity of this polymer at pH 5.0. Moreover, Lyness and Doelle[21] reported that pH 5 was optimum for levan production. They reported that pH was an important factor for the polysaccharide production, since high or very low values may repress levansucrase, the enzyme responsible for the biopolymer formation.

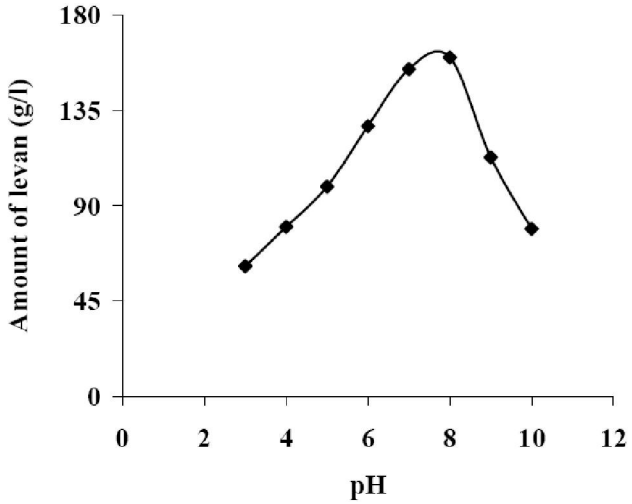

Figure 5

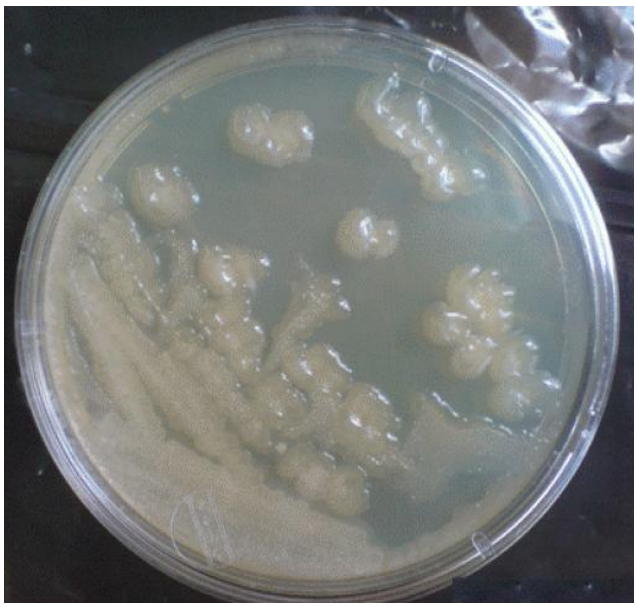

Figure 6 : Enzymatic synthesis of levan by *B.* licheniformis strain on agar plate with sucrose

Visual aspect of levan produced

The *Bacillus licheniformis* strain was grown on agar with sucrose. In the presence of sucrose, the colonies had a slimy mucoid appearance, which indicated the production of the polysaccharide from sucrose (Figure 6).

When ethanol was added in tube containing crude enzyme incubated with sucrose for 18h, a biphasic liquid/liquid batch reaction carried out in ethanol 2.5 v/v (Figure 7A). After a period of time levan starts to precipitate and a monophasic batch reaction appears (Figure 7B).

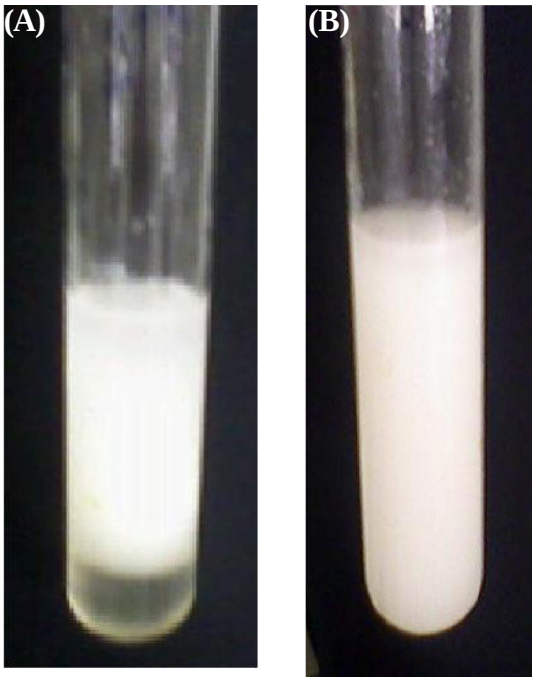

Figure 7 : Visual aspect of levan produced; Tubes showing: (A) a biphasic batch reaction carried out in ethanol 2.5 v/v, (B) a monophasic batch reaction after levan precipitation

CONCLUSION

The experiments showed that yeast extract does not affect levan production by *B. licheniformis*. However, Levan production is affected by initial concentration of sucrose, reaction time, temperature and pH in the experimental ranges studied. The results show that the enzyme can produce an important amount of levan up to 80 g/l of levan when a concentration of 300 g/l of sucrose was used at pH 8 at 50°C for a period of 18h.

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The obtained results are also important making levan different from the previously reported levans and can be successfully used in industrial applications.

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REFERENCES

[1] LViikari, R.Gisler; Applied Microbiology Biotechnology, **23**, 240- 244 **(1986)**.
[2] Y.Ben Ammar, T.Matsubara, M.Iizuka, T.Limpaseni, P.Ponsawasdi; Enzym.Microb.Technol., **30**, 875-882 **(2001)**.
[3] J.A.M.Vanbalken, J.G.M.Vandooren, W.J.J.Vandentweel, J.Kamphuis, E.Meijer; Applied Microbiology Biotechnology, **35**, 216-221 **(1991)**.
[4] Y.Yamamoto, Y.Akahashi, M.Kawano, M.Iizuka, T.Matsumoto, S.Saeki, H.Yamaguchi, Journal Nutrition Biochemical, **10**, 13-18 **(1999)**.
[5] H.Belghith, K.B.Song, C.H.Kim, S.K.Rhee, Biotechnol.Lett., **18**, 467-472 **(1996)**.
[6] V.K.Ananthalakshmi, P.Gunasekaran, Braz.Arch.Biol., **42**, 291-297 **(1999)**.
[7] J.Leibovici, Y.Stark, Cellular Molecular and Biology, **31**, 337-341 **(1985)**.
[8] V.Senthilkumar, N.Rameshkumar, S.J.W.Busby, P.Gunasekaran, Journal of Applied Microbiology, **96**, 671- 676 **(2004)**.
[9] K.H.Jang, K.B.Song, C.H.Kim, B.H.Chung, S.A.Kang, Biotechnol.Lett., **23**, 339-344 **(2001)**.
[10] K.S.Belghith, I.Dahech, H.Belghith, H.Mejdoub; International Journal of Biological Macromolecules, **50**, 451-458 **(2012)**.
[11] G.L.Miller, Analytical Chemistry, **31**, 426- 428 **(1959)**.
[12] I.Dahech, K.S.Belghith, H.Belghith, H.Mejdoub, Int.J.Biol.Macromol., **51**,329-335 **(2012)**.
[13] I.L.Shih, Y.T.Yu, C.J.Shieh, C.Y.Hsieh, Agric.Food Chem., **53**, 8211-5 **(2005)**.
[14] I.W.Sutherland, Carbohydrate Polymers, **38**, 319-328 **(1999)**.
[15] S.C.Park, J.Baratti, Applied Microbiology and Biotechnology, **38**, 542-549 **(1993)**.
[16] G.M.Vinhas, G.M.T.Calazans, C.E.Lopes, Paper present at SINAFERM, **(2000)**.
[17] F.Ahmed, Microbiology, **51**, 402-407 **(2005)**.
[18] U.Hettwer, M.Gross, K.Rudolph, J.Bacteriol, **177**, 2834-2839 **(1995)**.
[19] H.W.Doelle, L.Kirk, R.Crittenden, H.Toh, Critical Reviews in Biotechnology, **13**, 57-98 **(1993)**.
[20] M.Reiss, W.Hartmeier, Food.Biotechnol., **4**, 69-75 **(1990)**.
[21] E.W.Lyness, H.W.Doelle, Biotechnol.Lett., **5**, 345-350 **(1983)**.