# Levan production by isolated mutants of *Zymomonas mobilis*

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Abstract: *Levan is formed by transfructosilation reactions and is basically constituted by units of fructose. It is utilized as thickener in food industry and also used in medical and pharmaceutical areas. Levan can be synthesized by several groups of bacteria, among others, by the microorganism Zymomonas mobilis, in medium containing sucrose, yeast extract and mineral salts. The fermentation processes were carried out at 30 °C in Erlenmeyers flasks and in bench-top fermentor. The strain of Zymomonas mobilis was a mutant obtained by treatment of natural strain with NTG (N-methyl, N-nitro, N-nitrosoquanidine). The results show that this mutant can produce up to 42g/L of levana and high yeast extract concentrations can inhibit its synthesis.*

Key words: *levan, levansucrase, Zymomonas mobilis*

Resumo: *A levana, formada através de reações de transfrutosilação, constitui-se basicamente por unidades de frutose e encontra aplicação como espessante na indústria alimentícia, sendo utilizada ainda nas áreas médica e farmacêutica. Pode ser sintetizada por vários grupos de bactérias entre elas a Zymomonas mobilis em meio contendo sacarose, extrato de levedura e sais minerais. As fermentações foram conduzidas a 30 °C em frascos de Erleynmeyer e em fermentador de bancada. A Zymomonas mobilis estudada é um mutante obtido através de tratamento da cepa selvagem com NTG (N-metil N-nitro N-nitroso*

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*Guanidina). Os resultados mostram que este mutante pode produzir até 42g/L de levana e que concentrações altas de extrato de levedura podem inibir sua síntese.*

Palavras-chave: *levana, levansucarase, Zymomonas mobilis*

# 1 Introduction

Levan is a biopolimer with bonds $\beta(2\ 6)$ formed by transfructosilation reactions and it is a by-product of the alcoholic fermentation by *Zymomonas mobilis* as well as the sorbitol. Its molecular weight can reach values around 107 Da, corresponding approximately to 60,000 units of fructose (Viikari, L., Gisler, R., 1986).

*Zymomonas* are negative Gram bacteria, oxygen (Vinhas *et al.*, 2000) and low pH tolerant and are also easy to be manipulated genetically (Brock *et al.*, 1994). They have the metabolic way Entner-Doudoroff, what allows producing several acids from glucose and fructose (Dawes *et al.*, 1966).

The levan formation during the sucrose fermentation by *Zymomonas* is due to extracellular levansucrase presence, that has optimum temperature and pH of 35°C and 5.5 respectively, but its activity can also be found intracellular. This enzyme is identified as 2,6-$\beta$-fructan 6-$\beta$-fructosil-transferase, molecular weight of 48,000 and is produced by several bacteria as *Pseudomonas sp.*, *Acetobacter aceti*, *Aerobacter levanicum* (*Erwinia herbicola*), *Bacillus natto´s* and *Bacillus polymyxa*, being *Bacillus subtilis* and *Zymomonas mobilis* the most studied for this purpose (Hestrin *et al.*, 1943, Chambert & Gonzy-Tréboul, 1976; Cote, 1988; Kojima *et al.*, 1993).

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 (Viikari & Linko, 1986; Van Balken *et al.*, 1991).

Levan presents applications as a thickening agent and a stabilizer in the food industry, (Cote, 1988; Ananthalakshmy & Gunasekaran, 1999), besides its activity against tumor cells through modification of their membranes, increasing its permeability to cytotoxic agents and consequently facilitating these agents' action (Leibovici & Stark, 1985).

Other levan and fructose oligomer applications derive from its capacity to act as fibers, bringing good physiological and biochemical effects. Yamamoto *et al.* (1999) observed that fructose oligomers ingestion reduced the cholesterol and triglycerides levels in mice blood, but this observation must be checked in humans.

Senthilkumar *et al.* (2004) studied the disruption of the extracellular *Zymomonas mobilis* sucrase gene (Sac C) to improved levan production. A Sac C defective mutant of *Z. mobilis* obtained was employed in assays of fermentation, and also the levansucrase activity and the levan production were measured. In sucrose medium, the sac C mutant strain produced threefold higher levansucrase than the parent strain. The sac C mutant strain produced 21,1g/L of levan in 24 h in sucrose medium, compared to 15,5g/L in the parent strain.

The main purpose of this work was to obtain a mutant of the strain of *Zymomonas mobilis* by treatment of the natural strain with N-methyl N-nitro N-nitrosoquanidine and to study the capacity of this mutant to synthesize levan in high concentrations levels.

## 2 Material and methods

**Microorganism and culture conditions:** *Zymomonas mobilis* CCT 4494 acquired from the Tropial Foundation of Research and Technology "André Tosello" was maintained in medium MC containing (g/L): sucrose (50.0), yeast extract (10.0), $MgSO_4$ $7H_2O$ (0.5), $(NH_4)_2SO_4$ (1.0), $KH_2PO_4$ (1.0) and agar-agar (20.0), after incubation at 30°C for 24 hours. The culture was maintained at 4°C and transferred to new tubes every 20 days.

**Preparation of the pre-culture:** the microorganism was inoculated in the MC liquid and incubated at 30°C for 24 hours with an aliquot of 10% (v/v) transferred to the fermentation medium.

**Fermentation conditions in Erlenmeyer flasks:** the fermentations were carried out in Erlenmeyer flasks of 500mL for 16 hours at 30°C with 100mL of the fermentation medium, that contained besides $KH_2PO_4$ 1.0g/L and $MgSO_4$ $7H_2O$ 0.5g/L, sucrose, yeast extract and $(NH_4)_2SO_4$ in the studied concentrations. The initial pH was adjusted to 6.

**Selection of mutants:** ten mL of grown culture was centrifuged and the cellular mass was treated with NTG N-metil N-nitro N-nitrosoquanidine (NTG) for 45 minutes at 30°C in the concentration of 50mg/mL. The cells were washed with water or with the own fermentation medium, being cultured in plates with agar RS at 30°C for 24 hours.

**Effect of the sucrose and yeast extract concentration:** the concentrations studied were: 150 and 180g/L of sucrose, 5 and 10g/L of yeast extract and 0; 0.2; 0.4; 0.6; 0.8 and 1.0g/L of $(NH_4)_2SO_4$. Each flask was inoculated with 10% (v/v) of pre-culture as described above.

**Batch and Fed Batch Fermentation:** a fermentor Bioflo III with 4L of useful volume was used in order to study the influence of the pH control and feeding with constant agitation of 200 rpm. The fermentor was inoculated with 10% (v/v) of pre-culture as in the previous fermentations. The pH was controlled at 6.0.

**Analytic methods:** total reducing sugars (TRS) and reducing sugars (RS) were determined in the culture supernatant by Somogyi-Nelson method. The samples coming from the fermentation were centrifuged to separate cells of the culture supernatant, which were collected to determination of TRS, RS and levan. In the determination of TRS it was carried out the hydrolyze of the supernatant with HCl 1.0 N (100°C for 15 minutes), and after, it was refrigerated and neutralized with NaOH 1.0 N. The levan was quantified in the supernatant by precipitation with

ethanol 70%, being followed by a drying process in vacuum at 65°C until constant weight.

## 3 Results and discussion

**Selected mutant.**

Two mutants of *Zymomonas mobilis* were obtained after natural strain treatment with NTG, one of them obtained when the treat cells were washed with water (ZW) and another when the cells were washed with medium (ZM). The colonies of the mutant ZM had opaque aspect, but not so opaque as the natural strain, and the ZW which had translucent aspect. Table 1 shows that the mutant ZW produced 64% more levan than the ZM and 41% more than the natural strain in 24 hours of fermentation, reaching almost 43g/L. However if the fermentation time increased to 48 hours there was a reduction of the levan concentration produced by both mutants and by natural strain (data of natural strain are not shown), despite the high sugar concentration remaining in the media, that allows to conclude that the levan must have other function besides substrate reserve. This decrease in levan concentration was more accentuated in the ZM fermentation certainly because this mutant was more able to produce not only the enzyme that synthesizes but also the enzyme that hydrolyses the levan. The levan yield ($Y_{P/S}$) in the ZW fermentation was 44.29% and in the ZM fermentation 36.57% in 24 hours. While for natural strain of *Zymomonas mobilis* was 10%.

Workers (Yoshida *et al.*, 1990) report the levan production by mutant of *Zymomonas mobilis*, but they reached only 34 g/L using Erlenmeyer flasks.

| Time (h) | pH | | Levan | | RS | | TRS | |
|---|---|---|---|---|---|---|---|---|
| | ZM | ZW | ZM | ZW | ZM | ZW | ZM | ZW |
| 16 | 4.54 | 3.51 | 10.24 | 20.66 | 32.42 | 16.00 | 145.51 | 100.21 |
| 24 | 3.70 | 3.66 | 26.12 | 42.76 | 10.75 | 7.55 | 108.58 | 83.46 |
| 48 | 3.60 | 3.54 | 21.30 | 12.82 | 5.17 | 6.57 | 87.16 | 82.48 |

Initial sucrose concentration - 180 g/L. All concentration in g/L to natural strain of *Zymomonas mobilis* in 24 hours of fermentation and the levan production was 30,3 g/L.

Table 1 - Levan production from sucrose fermentation by mutants of *Zymomonas mobilis* CCT 4494. The initial pH was adjusted to 6.0. Reducing sugars (RS) and total reducing sugars (TRS).

**Effect of sucrose, yeast extract and $(NH_4)_2SO_4$ concentration on levan production.**

Table 2 shows the results of levan production by the mutant ZW in 16 hours of fermentation when sucrose, yeast extract and $(NH_4)_2SO_4$ concentration changed. In the medium with 150g/L of sucrose and 5g/L of extract yeast there was reduction of the levan produced when the $(NH_4)_2SO_4$ concentration increased, opposite behavior observed in the medium with 10 g/L of yeast extract, when the levan

concentration was higher with the increase of $(NH_4)_2SO_4$ concentration. Thus, the levan production is influenced not only by the carbon/inorganic nitrogen ratio but also by organic nitrogen/inorganic nitrogen ratio. The yeast extract analysis by Kjeldahl method (AOAC, 1995) showed that the extract had 10% of total nitrogen and as it is known, to be rich in vitamins and other compounds that have nitrogen besides some proteins that supply the mutant necessity. When the sucrose concentration increased to 180g/L, the levan production was higher than the production with 150g/L, but some specific relation to the levan production was observed with the $(NH_4)_2SO_4$ concentration increase, being higher the levan productions obtained when the medium had 180 g/L of sucrose, 0.2g/L of $(NH_4)_2SO_4$ and 5g/L of yeast extract (first fermentation) or when the medium had 180 g/L of sucrose, 0.6g/L of $(NH_4)_2SO_4$ and 10g/L of yeast extract (second fermentation). The remaining sugar in these two fermentation processes shows that in the first (batch fermentation), the levan yield was higher than in the second fermentation (fed batch fermentation), despite the sugar consumption had been higher in the second. This fact suggests that the maintenance energy spent by the mutant was higher in the second fermentation, nevertheless the method used to determine the levan concentration presented some difficulties, since it determines only the levan in supernatant of the culture, while some levan can be attached to the cell walls in capsule form. This levan capsules surrounding the cells are responsible for the difficulties of the microorganism growth in the pre-cultures because the cells do not recognize the medium in the environment and stay in latent state. Brock *et al.* (1994) observed that *Zymomonas mobilis* changes its Gram from negative to positive if it is cultivated during some time and such change is probably due to the attachment of the levan on its wall.

**Batch fermentation**

Figure 1 shows that the levan production reached only 30.98g/L when the fermentation was carried out in fermentor Bioflo III, without aeration, using the better medium defined in Table 2 (sucrose - 180g/L, yeast extract - 5g/L and $(NH_4)_2SO_4$ - 0.2g/L), that it is practically the same levan concentration obtained in the Erlenmeyer flasks fermentation (Table 2) of the 30.32g/L, but the TRS consumption in fermentor was 83.43% and in Erlenmeyer flasks 47.41%. The fall in the levan yield allowed to suppose that oxygen had some negative influence on the levan production, as reported by Vinhas *et al.* (2000) who obtained the highest yield using Erlenmeyer flasks without agitation. The *Zymomonas mobilis* is known as an anaerobic microorganism that tolerates oxygen, therefore we decided to carry out another fermentation with aeration of 2vvm, which results are also shown in Figure 1 and 2. These figures prove the suspicion that oxygen has influence on the levan production and the fall of the levan yield in the fermentor was due to the power input (200rpm) that promotes the oxygen of the head space dissolution in the medium, while in Erlenmeyer flasks the oxygen dissolution is not so intense, that allows to obtain results like shown in Table 1 (42.76g/L). A suggestion to avoid the yield fall would be to introduce a nitrogen flow rate through the free space of the fermentor during the fermentation to assure anaerobic condition.

The production of RS in the experiments without aeration started after 4 hours of fermentation (Figure 2) and the levan production started after the decrease of RS concentration as a consequence of fructose polymerization by levansucrase activity, but in the experiment with aeration RS started immediately after inoculation without levan production.

| Sucrose | Yeast Extract | $(NH_4)_2SO_4$ | pH | TRS | RS | Levan |
|---|---|---|---|---|---|---|
| 150 | 5 | 0.0 | 4.07 | 50.23 | 16.50 | 22.78 |
| | | 0.2 | 4.00 | 120.27 | 26.88 | 21.08 |
| | | 0.4 | 4.07 | 96.51 | 27.21 | 18.32 |
| | | 0.6 | 3.82 | 75.22 | 23.35 | 20.74 |
| | | 0.8 | 4.00 | 91.59 | 27.49 | 18.68 |
| | | 1.0 | 3.32 | 53.43 | 9.19 | 19.82 |
| 150 | 10 | 0.0 | 4.54 | 27.21 | 3.94 | 13.20 |
| | | 0.2 | 4.54 | 66.72 | 15.63 | 16.14 |
| | | 0.4 | 4.47 | 67.46 | 17.40 | 15.80 |
| | | 0.6 | 4.45 | 64.38 | 19.24 | 17.26 |
| | | 0.8 | 4.35 | 42.10 | 7.10 | 18.40 |
| | | 1.0 | 3.72 | 65.00 | 10.75 | 21.36 |
| 180 | 5 | 0.0 | 3.76 | 85.93 | 27.70 | 27.30 |
| | | 0.2 | 3.51 | 94.67 | 27.41 | 30.32 |
| | | 0.4 | 3.35 | 98.11 | 16.50 | 23.12 |
| | | 0.6 | 3.39 | 102.05 | 13.30 | 20.06 |
| | | 0.8 | 3.39 | 96.51 | 12.43 | 19.24 |
| | | 1.0 | 3.36 | 70.05 | 11.16 | 25.38 |
| 180 | 10 | 0.0 | 4.03 | 51.58 | 13.95 | 21.60 |
| | | 0.2 | 4.18 | 148.34 | 29.38 | 18.30 |
| | | 0.4 | 3.84 | 111.41 | 33.28 | 26.94 |
| | | 0.6 | 3.71 | 83.22 | 20.43 | 27.14 |
| | | 0.8 | 3.62 | 63.52 | 19.45 | 24.12 |
| | | 1.0 | 3.58 | 50.23 | 7.14 | 25.02 |

All concentration in g/L.

Table 2 - Effect of the sucrose, yeast extract and $(NH_4)_2SO_4$ concentration in the levan formation by *Zymomonas mobilis*, concentration of Reducing Sugars (RS) and Total Reducing Sugars (TRS), in 16 hours of fermentation.

**Fed Batch Fermentation.**

Figure 3 shows the results of a fed batch fermentation carried out as a tentative to increase the levan production. The initial volume of medium in the fermentor was 2L and the feeding began in the fourth hour after inoculation and was stopped in the eighth hour when the RS production and levansucrase activity were intense, but contrary to the expected, the levan production reached only up to 7g/L.

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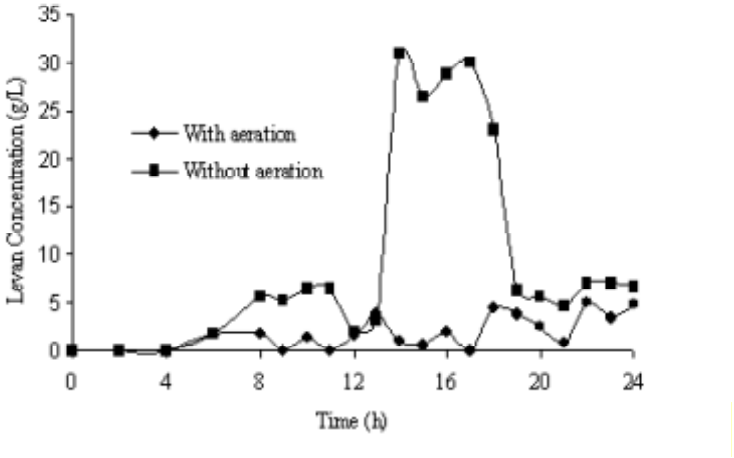


Figure 1 - Levan production by Zymomonas mobilis with and without aeration.

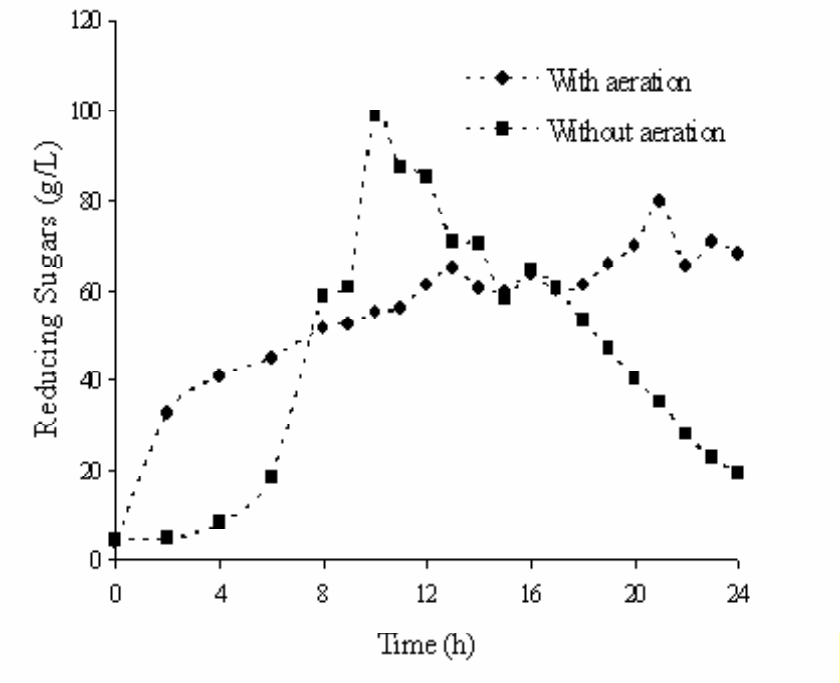


Figure 2 - The production of Reducing Sugars in the experiments with and without aeration.

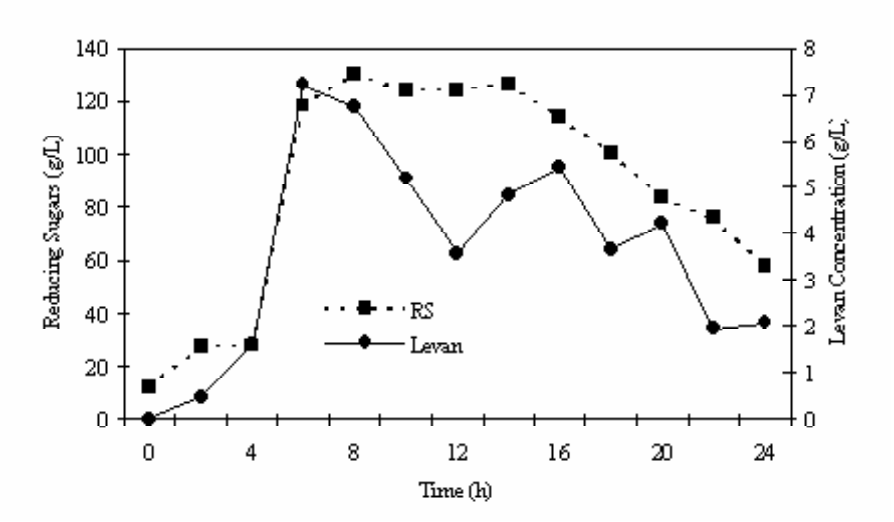


Figure 3 - Results of a fed batch fermentation, levan production (g/L) and Reducing Sugars (g/L).

We conclude that to obtain mutants from *Zymomonas mobilis* is easy, which can increase the levan production reaching concentration of 40g/L, but this process must be better studied. The reproduction of the results from experiments carried out in Erlenmeyer flasks or fermentor is difficult since high oxygen concentration in the medium inhibits levan synthesis, being necessary to maintain anaerobic condition to increase its yield. In this work it was possible to observe that the cells had high specific weight and settled rapidly, which was another fact that allowed to conclude that the cells were encapsulated with levan. If this levan from cell walls could be extracted, or if the encapsulation could be avoided, the levan yield could be better.

Unfortunately the levan production occurs when the initial concentration of sucrose in the fermentation medium is high, remaining high concentrations of TRS. If the fermentation is carried out for a long time, the levan can be hydrolyzed. However, in this fermentation there is ethanol production that must be recovered.

The fed batch fermentation did not present advantage over batch fermentation, but several forms of this fermentation can be tried to increase the levan yield, for example, feeding the fermentor high flow rate and different sucrose concentration.

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