

# Mutagenesis of *Leuconostoc dextranicum* NRRL B-1146 for higher glucan production

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## Abstract

The constitutive mutants of *Leuconostoc dextranicum* NRRL B-1146 were generated by classical mutagenesis technique using UV radiation for higher production of glucan. The conditions of mutagenesis such as dilution factor and time of UV light exposure were optimized. Three stage screening of mutants was carried out and the mutants were grown and subsequently tested for glucan content produced after the second stage. In the first stage 137 mutants were picked by visual screening based on morphology and colony size and grown. In the second stage of screening, 97 colonies were visually screened from 137 colonies and glucan content was analysed by microtitre format. In the third stage screening, 11 higher glucan producing mutants were selected from 97. Finally, 3 mutants produced significantly high glucan content, out of 11 selected mutants. The wild-type strain gave 1.01 g/l of glucan using statistically optimized medium. The mutant 64 gave maximum glucan content of 5.1 g/l, 5 times higher than that produced by wild-type *Leuconostoc dextranicum* NRRL B-1146 followed by mutant 88 giving 4.24 g/l and mutant 9 giving 4.1 g/l.

## INTRODUCTION

Glucanases are extracellular proteins involved in the synthesis of  $\beta$ -glucans. Lactic acid bacteria produce a wide variety of exopolysaccharides [1,2]. *Leuconostoc* species are commercially exploited for the production of glucans, homopolysaccharides which contain only one type of monosaccharide, glucose. Glucans differ in the type of glucosidic linkages, degree and type of branching, length of glucan chains, molecular mass and conformation of polymers [3]. All these properties strongly contribute to specific polysaccharide properties such as solubility, rheology and other physical characteristics. Depending on the main chain glucosidic linkages in glucan, three different types of  $\beta$ -glucans synthesized by *Leuconostoc* are known viz. dextran with  $\beta$ -(1-6), mutan with  $\beta$ -(1-3) and alternan with  $\beta$ -(1-6),  $\beta$ -(1-3) linkages [3]. Chemical modifications in polysaccharides have been extensively studied and reviewed [4]. Glucans are potential therapeutic agents and are also used as viscosifying, stabilizing, emulsifying, sweetening, gelling, or water-binding agents, in the food as well as in non-food industries [5,6].

The efficiency of established bioprocesses can be increased either by introducing more productive strains or by optimizing the cultivation medium itself [7]. Development of new functional polysaccharides or glucans is important

for food industry [8]. Classical mutagenesis techniques can be used to generate mutants for improvement of enzyme and or glucan production. It can lead to production of new types of glucans. The selection for the desired strain and hunting for mutants however is cumbersome. Efficient mutagenesis and good planning pays off in time and labor. Mutants of *Leuconostoc* strains NRRL B-512F [9], B-742 [10] and B-1299 [11] and 512FMC [12] are presently used in the industry for their traits. The traditional approach of creating mutants is by exposing the microbes to ultraviolet (UV) light that is fairly efficient in producing substitutions. The glucan produced by *Leuconostoc dextranicum* NRRL B-1146 is unique with  $\beta$ -(1-6) and  $\beta$ -(1-4) linkages [7] and also contains viscosifying and gelling properties [13]. The present study describes classical mutagenesis of *Leuconostoc dextranicum* NRRL B-1146 by UV irradiation technique, generating mutant strains with enhanced glucan production.

## MATERIALS AND METHODS

### MUTAGENESIS OF BY ULTRA VIOLET (UV) IRRADIATION

*Leuconostoc dextranicum* NRRL B-1146 was grown (at 28°C at 200 rpm) in 10 ml of statistically designed liquid medium as described earlier by Majumder et al. 2009 [7]. A cell concentration of  $5.2 \times 10^7$  cfu/ml was used and 1 ml of these cells were serially diluted in 9 ml sterile saline (0.85%)

contained in test tubes. 100  $\mu$ l from each of the tubes with dilution factor of  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  was plated on modified MRS agar medium described by Goyal and Katiyar, 1996 [14] contained in the 90 mm Petri dishes. The Petri dishes were exposed to UV light for different time intervals (1, 2 and 3 min) to determine the percent survival of cells. Percent survival of colonies was calculated as the number of colonies survived after UV light exposure over the total number of colonies in the control, multiplied by 100. Immediately, after UV light exposure, the Petri dishes were kept in the dark for 2h in order to avoid photo-reactivation. A control for each dilution was used that was not exposed to UV light. The percent survival of the cells on Petri dishes was calculated at different UV exposure durations and at different dilution factors with respect to the controls. The colonies were picked at the end of 48 h based on the morphology, size and growth rate of colony. The individual colonies were picked and inoculated in to 10 ml liquid medium of Majumder et al., 2009 [7] to test for glucan production. The UV irradiation was chosen to give 0.1 - 2% survivors for further experiments.

## GLUCAN ESTIMATION

The wild-type *Leuconostoc mesenteroides* NRRL B-1146 and mutants were grown in 10 ml of statistically optimized liquid medium of Majumder et al. 2009 [7] at 28°C and 200 rpm. 1 ml samples were taken at 48h from the fermenting broth for glucan estimation. The time period of 48 h was optimum for glucan production as reported earlier [7]. After centrifugation the cell free supernatant was analyzed for glucan content. For determination of glucan content 200  $\mu$ l supernatant was treated with two volumes of ethanol at 4°C for 1h and centrifuged at 10,000g for 10 min. The precipitate obtained was dissolved in an equal amount of water and the glucan content was estimated according to method of Fox and Robyt, 1991 [15] taking the 10 kDa dextran as standard. All assays were performed using microtiter formats, which is an efficient method for screening of mutants producing higher amounts of glucansucrase or glucan. This method helps in handling and screening large number of colonies, reduces the work load, time required and also the volume of the sample and reagents used.

## RESULTS AND DISCUSSION

The grown *Leuconostoc dextranicum* NRRL B-1146 cells on Petri dishes using  $10^{-3}$  -  $10^{-6}$  dilutions were exposed to UV radiation for 1, 2 and 3 min time duration. A control for each dilution was used that was not exposed to UV light. The percentage survival of the cells on Petri dishes was

calculated at different exposure times and different dilutions with respect to the controls. It was found that the percent survival was optimum at an UV light exposure time of 1 min and at dilution factors  $10^{-5}$  and  $10^{-6}$ . No colony appeared on Petri dish with dilution factor of  $10^{-3}$  and only 0.08% colonies survived on  $10^{-4}$  dilution factor Petri dishes for 1 min UV light exposure. The Petri dishes with 0.1 to 2% cell survival rates were selected. No colony grew on Petri dishes with 2 and 3 min UV exposure at any dilution. The mutagenesis by UV irradiation was optimised at 1 min and at dilution factors  $10^{-5}$  and  $10^{-6}$  and the conditions were used for further experimentation.

Under the optimised mutagenesis conditions of dilution factor of  $10^{-5}$  and  $10^{-6}$  with 1 min duration of UV light exposure, 13 Petri dishes for each dilution factor were used in order to get sufficient number of mutants. After the UV light exposure, the clusters of colonies that grew around the edges of the Petri dishes were not picked and only those that grew at the centre of Petri dishes were taken (Table 1). From the Petri dishes of dilutions  $10^{-5}$  and  $10^{-6}$ , 137 colonies were visually screened based on their morphology and size and further grown on modified MRS agar Petri dishes. From 137 grown colonies, 97 mutants were again visually screened and glucan content of all was estimated using the microtitre format. From the analysis of the glucan produced by 97 mutants, 11 hyper-producer mutant strains were selected.

**Figure 1**

Table 1. Number of colonies after UV irradiation of Petri dishes for 1 min with  $10^{-5}$  and  $10^{-6}$  dilution factors.

	No. of colonies		
	Dilution factor $10^{-5}$	Dilution factor $10^{-6}$	Total
Control	960	320	1280
Obtained after UV light exposure	131	87	218
Selected	68	69	137

The Figure 1 shows the glucan content of each of these 11 hyper-producer mutant strains. The wild-type *Leuconostoc dextranicum* NRRL B-1146 as control gave 1.01 g/l of glucan content in our earlier report [7] that also served as a control in the present study. The numerical data for glucan content of 11 mutants and fold increase are reported in Table 2. The mutant 64 gave maximum glucan content of 5.1 g/l, followed by mutant 88, giving 4.2 g/l and then mutant 9, giving 4.1 g/l (Table 2). It was observed that the mutant nos. 9, 64 and 88 showed very high glucan production, while mutant nos. 10, 58, 63, 74, 82 and 84 showed an appreciable increase of glucan production (Table 2, Fig. 1). The two

mutants viz. 24 and 91 gave insignificant increase in glucan content upon repeated estimations, even though they showed a higher concentration when the test was earlier performed in the micro-titre format. This was attributed to the instability of the mutants. The present results of mutant no. 64 generated by mutagenesis of the wild-type *Leuconostoc dextranicum* NRRL B-1146 showed a further 5 times increase of glucan production to 5.1 g/l.

Figure 2

Fig . 1: Glucan production by 11 hyper producer mutants and wild-type strain.

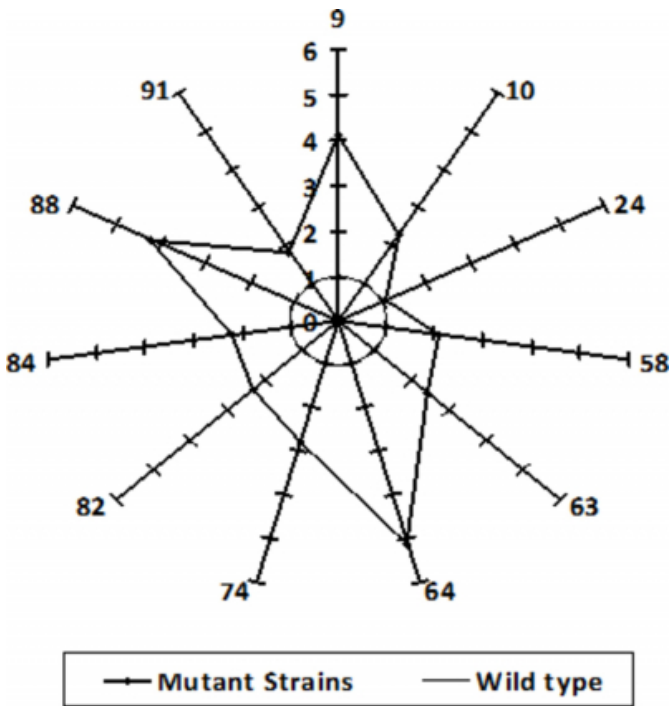
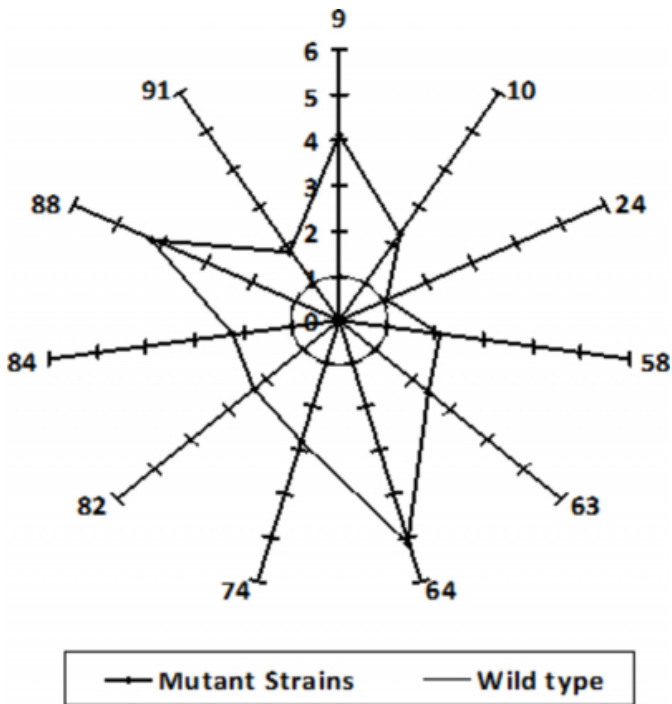


Figure 3

Table 2. Glucan content of 11 mutants selected from 97 mutants



CONCLUSIONS

The wild-type *Leuconostoc dextranicum* NRRL B-1146 was subjected to mutagenesis by UV radiation to generate mutants that produced higher glucan. The conditions for mutagenesis were optimized at  $10^{-5}$  and  $10^{-6}$  dilution factors with an exposure time of 1 min. In the first stage of screening after UV treatment, 137 colonies were picked based on visual analysis of colony morphology and were grown. In the second stage of screening, from 137 mutants about 97 were picked by visual screening on the basis of rapid growth and a visible sheen indicative of the glucan produced. These 97 mutants were then tested for glucan content using a micro-titre based rapid assay. In the third stage of screening of mutants, from the 97 mutants 11 higher glucan producing and fast growing strains were identified. These 11 mutants were then tested for stability by repeatedly sub-culturing every 15 days over a period of 6 months. They were found to be stable with no observable change in the characteristics and concentration of the glucan produced for most of them. They were then again tested for glucan content and it was found that of the 11 mutants, 3 were very high glucan producing mutants, 8 were higher than the wild type strain's production of glucan. The mutant number 64 gave maximum glucan concentration of 5.1 g/l which is 5 times higher than that produced by wild-type *Leuconostoc dextranicum* NRRL B-1146 that gave 1.01 g/l glucan. The

results showed that the classical mutagenesis technique of UV irradiation improved the strain by increasing its growth rate or by increasing the amount or the activity of enzyme release causing higher production of glucan.

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