Stability Study of Crude Dextranucrase from *Leuconostoc citreum* NRRL B-742

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Abstract In the present work, the stability of crude dextranucrase from *Leuconostoc citreum* B-742 was evaluated in synthetic and in cashew apple juice culture broth. Optimum stability conditions for dextranucrase from *L. citreum* B-742 were different from the reported for its parental industrial strain enzyme (*L. mesenteroides* B-512F). Crude dextranucrase, from *L. citreum* B-742, produced using cashew apple juice as substrate, presented higher stability than the crude enzyme produced using synthetic culture medium, showing the same behavior previously reported for dextranucrase from *L. mesenteroides* B-512F. The crude enzyme presented good stability in cashew apple juice for 48 h at 25°C and pH 6.5.

Keywords Agriculture substrates · Enzyme stability · Glycosyltransferase · Fermentation · *Leuconostoc citreum*

Introduction

Free enzymes in crude fermented broth are usually unstable and must be stabilized just after the production. Costly purifications protocols are often employed making enzyme process expensive for large scale application. To reduce the process costs, enzyme immobilization has been studied by several authors due to its reutilization for several times. Dextranucrase is considered an enzyme difficult to immobilize because of its natural association with native dextran. Some time ago the only available technique for

dextranucrase immobilization was the entrapment in alginate matrix. However, this difficult has been overcome by using dextranases and new immobilization strategies and supports [1–4].

Leuconostoc citreum NRRL B-742, former named *L. mesenteroides* B-742, is a lactic acid bacterium able to produce dextranucrase and prebiotic oligosaccharides [5–7]. Despite the ability of producing prebiotic oligosaccharides, few studies have been published with this strain and the most studied strain for dextranucrase production is *Leuconostoc mesenteroides* B-512F [8–10]. Dextranucrase from *L. mesenteroides* B-512F synthesizes linear oligosaccharides while the enzyme obtained from *L. citreum* B-742 synthesizes branched oligosaccharides [5].

Alternative substrates have been studied as low cost substrates for microbial fermentation to produce several products for chemical, petrochemical, pharmaceutical and food industry [11–16]. Agriculture residues and wastes are the most suitable low cost substrates for microbial cultivation.

Cashew apple is the peduncle of the cashew fruit, which is rich in reducing sugars (fructose and glucose), vitamins, minerals and some amino acids. The cashew tree grows even on poor soils with low rainfall and it is cultivated in 32 countries around the world and Brazil, India, Vietnam and Nigeria are the main producers. Although cashew apples can be consumed as juice, ice-cream and other foodstuffs, the cashew tree cultivation in Brazil is an activity that aims mainly to produce cashew nuts. The nuts represent only 10% of the total fruit weight, and large amounts of cashew apples are left in the field after the removal of the nut [17].

According to official data, the Brazilian Northeast presents an annual production of approximately 2 millions of tons of cashew apples and 90% of this production is lost or

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underutilized. As such, the cashew apple is considered an agriculture waste and its nutritive juice can be a suitable low cost substrate for biotechnological process. Cashew apple juice is rich in fructose, glucose and minerals. These sugars can act as acceptors to produce oligosaccharides using glycosyltransferases enzymes such as dextranucrase [17, 18].

The goal of the present work was the evaluation of the stability of dextranucrase from *Leuconostoc citreum* B-742 in crude fermented broth. Enzyme stability is a key factor before considering industrial applications for enzyme process development because crude enzymes in the fermented broth are usually easily denaturated.

Materials and Methods

Raw Material

Cashew apple (*Anacardium occidentale* L.) juice was obtained through mechanical process. The juice, which contains high levels of tannins, was clarified by adding gelatin to remove tannins and suspended solids [17]. The clarified cashew apple juice was physical-chemically characterized, centrifuged at $11,806\times g$ for 10 min and stored frozen (-20°C) prior to use.

Juice Characterization

Total reducing sugar was measured by the DNS method [19] using a UV–visible spectrophotometer. The pH was determined by direct measure in a Marconi PA 200 potentiometer.

Dextranucrase Production

A glycerol stock culture of *Leuconostoc citreum* B-742 obtained from ARS Culture Bacterial Collection (NRRL Culture collection, United States Department of Agriculture, Peoria, Illinois -USA) was activated in an optimized synthetic medium composed of sucrose, 50 g/l (food grade); yeast extract, 20 g/l (Himedia); $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.20 g/l; $\text{MnSO}_4\cdot 2\text{H}_2\text{O}$, 0.01 g/l; $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, 0.01 g/l; $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 0.02 g/l; NaCl, 0.01 g/l; and K_2HPO_4 (anhydrous), 20 g/l [9, 20, 21] in an orbital shaker at 30°C at 150 rpm for 12 h. Ten percent of this cell culture was used as inoculums to the fermentation medium for enzyme production.

Dextranucrase was produced in a culture medium composed by cashew apple juice in substitution to the synthetic culture medium and in the synthetic medium optimized by Rabelo [7]. The cashew apple juice culture medium was prepared by diluting the clarified apple juice

to reach 50 g/l of reducing sugar content and by adding sucrose (50 g/l), yeast extract (4 g/l); ammonium sulfate (4 g/l) and K_2HPO_4 (20 g/l). No minerals salts were added to the juice because it is rich in minerals [17, 22]. The synthetic culture medium had the same composition of the activation medium with yeast extract content changed by using a mixture containing 4 g/l of extract and 4 g/l of ammonium sulfate [7].

Sucrose was added to induce the dextranucrase production. The initial pH was adjusted to 6.5 and the culture medium was sterilized at 121°C for 15 min. Fermentation was carried out in 250 ml Erlenmeyer's flasks containing 100 ml of the culture medium in an orbital shaker at 30°C and 150 rpm of agitation. The process was interrupted when the medium pH reached 5.5, to avoid enzyme denaturation. After harvesting the cells by centrifugation at $11,806\times g$ (8,000 rpm) for 10 min, the supernatant was used in the crude enzyme stability assays.

Enzyme Activity

The substrate for enzyme activity assay was a 10% (p/v) sucrose solution dissolved in a 20 mM sodium acetate buffer at pH 6.5 containing 0.05 g/l of CaCl_2 [7]. Enzyme activity was determined by quantifying the initial rate of reducing sugar released by DNS method [14]. Dextranucrase promotes the sucrose cleavage polymerizing the glucose into dextran chain and releasing the fructose moiety [9, 20, 21].

The crude fermented broth (100 μl) incubated with 400 μl of the substrate solution at 30°C for 10 min [7]. Results were expressed in IU/ml where 1 IU is the amount of enzyme that releases 1 $\mu\text{mol min}^{-1}$ of reducing sugar at the assay conditions.

Enzyme Stability Assay

Stability assays were carried out with crude cashew apple fermented broth and with crude synthetic fermented broth. The stability of the crude enzyme produced in the synthetic medium was assayed according to a factorial planning where temperature and pH were changed in the range of $25\text{--}35^{\circ}\text{C}$ and 4.5–6.5, respectively (Table 1). Sodium azide (0.01% w/v) was added to the crude fermented broth to avoid microbial contamination. Crude enzyme fermented broth aliquots (20 ml) had their pH values adjusted to the desired pH with NaOH or H_3PO_4 . The initial activity for each pH value was determined as previously described. Stability assay was carried out for period of 3 h.

The stability of the crude enzyme produced in the cashew apple medium was assayed near to the best condition found for the synthetic medium (pH 6.5 and temperature of 30 and 35°C). Sodium azide (0.01% w/v) was

Table 1 Enzyme activity of crude dextranucrase produced in synthetic medium as function of pH and temperature at 0.5 and 1 h of stability assay

Assay	pH	Temperature (°C)	Enzyme activity (DSU/ml)	
			0.5 h	1 h
1	4.5	25	0.22 ± 0.03	0.25 ± 0.05
2	4.5	35	0.33 ± 0.08	0.45 ± 0.01
3	6.5	25	1.01 ± 0.05	0.70 ± 0.07
4	6.5	35	0.66 ± 0.06	0.48 ± 0.02
5	5.5	30	0.56 ± 0.09	0.47 ± 0.05

added to the crude fermented broth to avoid microbial contamination. Crude enzyme fermented broth pH was adjusted to 6.5 with NaOH. The stability assay was carried out for a period of 48 h. Longer assay period was considered for the enzyme in cashew apple juice crude fermented broth due to the high stability at room temperature (30°C) previously reported for dextranucrase from *L. mesenteroides* B-512F produced in the same substrate.

Statistical Analyses

Results are shown as means ± SD. Statistica 7.0 (Statsoft) was used to process the data.

Results and Discussion

The experiments were carried out in duplicate, and the assays were done in triplicate. Results are presented as mean value ± SD. The pH of clarified cashew apple juice used in the present study was 4.3 and the juice presented 91.59 g/l of reducing sugar. Enzyme stability in the crude synthetic fermented broth was assayed during 3 h with the environmental conditions (pH and temperature) changing according to the experimental design presented in Table 1. The results are presented in Tables 1, 2. Estimated effects of the independent variables on dextranucrase activity are presented in Tables 3, 4 at the given sampling intervals.

Table 2 Enzyme activity of crude dextranucrase produced in synthetic medium as function of pH and temperature at 2 and 3 h of stability assay

Assay	pH	Temperature (°C)	Enzyme activity (DSU/ml)	
			2 h	3 h
1	4.5	25	0.15 ± 0.02	0.13 ± 0.02
2	4.5	35	0.30 ± 0.07	0.23 ± 0.01
3	6.5	25	0.70 ± 0.09	0.61 ± 0.05
4	6.5	35	0.50 ± 0.05	0.35 ± 0.02
5	5.5	30	0.41 ± 0.01	0.31 ± 0.05

Table 3 Estimated effects of pH and temperature on dextranucrase from *L. citreum* B-742 activity

Factor	0.5 h		1 h	
	Effect	S.E.	Effect	S.E.
Mean	0.558*	0.002	0.469*	0.001
pH	0.558*	0.004	0.242*	0.003
Temperature	−0.123*	0.004	−0.016	0.003
pH × Temperature	−0.232*	0.004	−0.0210*	0.003

* Significant at 95% of confidence level

Table 4 Estimated effects of pH and temperature on dextranucrase from *L. citreum* B-742 activity

Factor	2 h		3 h	
	Effect	S.E.	Effect	S.E.
Mean	0.412*	0.002	0.328*	0.010
pH	0.380*	0.006	0.299*	0.027
Temperature	−0.025	0.006	−0.086	0.027
pH × Temperature	−0.176*	0.006	0.183	0.027

* Significant at 95% of confidence level

According to the results presented on Table 3, all variables presented statistically significant effect on enzyme activity at 0.5 h of stability assay. The effect of temperature was negative and the effect of pH was positive. On the other hand, the interaction of pH and temperature presented negative effect on enzyme stability. At 1 h of the stability assay, temperature did not present significant effect and pH presented a lower effect on enzyme activity than the observed at 0.5 h of assay. Temperature and pH interaction presented negative effect on enzyme activity. At 2 h of assay (Table 4) the same behavior was observed, however pH effect on enzyme stability was higher than the obtained at 1 h of assay. This increase followed by decrease in the activity value may be attributed to enzyme conformation stabilization along the time because the temperature was significant only at the beginning of assay. At 3 h of assay, only the pH and the mean were statistically significant.

Regression models for the enzyme activity for each time of sampling are presented in Eqs. 1–3. ANOVA tables and model validation for these fitted models are summarized on Table 5. The fitted regression model obtained for 3 h of stability assay (not presented) was not statistically significant.

$$\text{Activity}_{0.5h}(\text{IU/ml}) = -4.43 + 0.97 \text{ pH} + 0.12 \text{ T} - 0.02 \text{ pH} \cdot \text{T} \quad (1)$$

$$\text{Activity}_{1h}(\text{IU/ml}) = -3.61 + 0.75 \text{ pH} + 0.11 \text{ T} - 0.02 \text{ pH} \cdot \text{T} \quad (2)$$

$$\text{Activity}_{2h}(\text{IU/ml}) = -3.46 + 0.71 \text{ pH} + 0.09 \text{ T} - 0.02 \text{ pH} \cdot \text{T} \quad (3)$$

Table 5 ANOVA analysis for the regression models (Eqs. 1–3)

Variation source	Quadratic sum	Degrees of freedom	Mean square	<i>F</i> -value
Activity at 0.5 of assay				
Regression	0.3798	3	0.1266	9738
Error	1.3×10^{-5}	1	1.3×10^{-5}	
Total		4		
R^2	0.9999			
<i>F</i> -listed (95%)				$F_{3,1} = 215.71$
Activity at 1 h of assay				
Regression	0.1029	3	4.42×10^{-2}	3117
Error	1.1×10^{-5}	1	1.3×10^{-5}	
Total	0.1029	4		
R^2	0.9999			
<i>F</i> -listed (95%)				$F_{3,1} = 215.71$
Activity at 2 h of assay				
Regression	0.1762	3	5.87×10^{-2}	1835
Error	1.1×10^{-5}	1	3.2×10^{-5}	
Total		4		
R^2	0.9998			
<i>F</i> -listed (95%)				$F_{3,1} = 215.71$

where pH is the pH of activity solution and T is the assay temperature (°C)

According to the ANOVA analysis and *F*-test, the considered fitted models were statistically significant at the given confidence interval because the calculated *F*-values were higher than the listed ones [10]. The results presented on Tables 1, 2 were analyzed by the response surface graphs presented in Figs. 1, 2, 3. According to them, pH has a strong effect on enzyme activity and the higher the pH the higher the enzyme activity. Temperature presented lower influence on enzyme activity. However, the interaction of temperature and pH was significant and the maximum enzyme activities were found in the region of 25°C and pH of 6.5 (around the conditions of the experimental run 3). Figure 4 present the enzyme relative activity calculated dividing the enzyme activity by the initial activity measured just after the crude fermented broth pH adjustment ($t = 0$).

According to the results presented in Fig. 4, run 1 presented an enzyme activity loss of 50% after 2 h of assay. Run 2 showed an activity increase for 1 h of stability assay followed by a decrease for 2 and 3 h. Runs 3 and 5 presented activity decrease at 1 and 2 h of assay followed by an increase for 3 h of assay. Run 4 showed enzyme stability loss after 1 h of assay. According to the results presented in Fig. 4, the crude enzyme was stable for at least 3 h (relative activity ≥ 1.0) at pH 6.5. At this pH, assays carried out at 25°C presented the highest relative activities. However, good enzyme activities were also found at 30°C and pH 6.5

Fig. 1 Surface response graph of dextranucrase activity as function of pH and temperature after 0.5 h of crude enzyme stability assay in synthetic medium

according to Figs. 1, 2, 3. From this results, stability assay for the enzyme produced using cashew apple juice as substrate was carried out at pH 6.5 and temperatures of 25 and 30°C. As previously reported, longer time intervals were considered for the enzyme stability of cashew apple crude enzyme due to the higher enzyme stability observed for dextranucrase from *L. mesenteroides* B-512F in this

Fig. 2 Surface response graph of dextranucrase activity as function of pH and temperature after 1 h of crude enzyme stability assay in synthetic medium

Fig. 3 Surface response graph of dextranucrase activity as function of pH and temperature after 2 h of crude enzyme stability assay in synthetic medium

substrate [17]. Figure 5 presents the results obtained as relative activity. Crude enzyme in synthetic culture medium was completely denaturated after 20 h of assay, thus results for this enzyme were not shown.

According to the results presented in Fig. 5, at 25°C the relative activity increased until 20 h of assay showing a decrease after this period, keeping this decrease until 48 h of the stability assay. Relative activity at this temperature

Fig. 4 Relative activity of crude dextranucrase after 3 h of stability assay in synthetic medium

Fig. 5 Relative activity of crude dextranucrase after 4 h of stability assay in cashew apple juice medium

increased up to four times and after 48 h of assay the relative enzyme activity was still higher than the initial. Similar behavior was found for the assay at 30°C. However, the relative activity values at 25°C were higher than the obtained at 30°C, especially considering the enzyme stability for more than 20 h. At 30°C the enzyme showed good stability up to 3 h of assay and the enzyme activity strongly decreased after that, with a strong decrease between 20 and 24 h of assay. After 48 h of assay, the enzyme was completely denaturated.

The results reported herein are in agreement to the results previously reported [17] for dextranucrase from *L. mesenteroides* B-512F stability in cashew apple juice. However, dextranucrase from *L. mesenteroides* B-512F presented higher stability than the enzyme studied herein.

On the other hand, *L. citreum* showed different stability conditions from its parental strain (*L. mesenteroides* B-5412F), which presented optimum stability at pH 5.2 and 30°C.

The results suggest that at least one component of cashew apple juice has a positive impact on dextranase structure, preserving its stability for a long time at temperatures that are considered high for enzyme storage (25°C) in crude fermented broth. For industrial applications, these results are very interesting because enzyme purification and stabilization are expensive steps. As an agriculture substrate, cashew apple juice presents complex composition, and besides glucose, fructose, and mineral salts, the juice also presents other compounds such as organic acids (mainly ascorbic and citric), carotenoids, and phenolics. These compounds might interfere in the enzyme structure, affecting and stabilizing its activity. The enzyme stabilization mechanism in cashew apple juice is still not known and may be caused by a specific juice component or by the combination of several components of the juice acting synergistically.

The increase and decrease on enzyme activity observed in Figs. 4, 5, was already reported [17] for dextranase from *L. mesenteroides* B-512F in crude cashew apple fermented broth. Girard and Legoy [23] also reported a similar behavior for commercial lyophilized dextranase from *L. mesenteroides* B-512F in several organic solvents. According to the authors, the increase followed by the decrease of the enzyme activity was caused by changes on the enzyme conformation in such solutions. In the present work, such variations may be attributed to the changes in the pH and/or temperature.

Conclusions

Optimum stability conditions for dextranase from *L. citreum* B-742 were different from the reported for its parental industrial strain enzyme (*L. mesenteroides* B-512F). Crude dextranase from *L. citreum* B-742 produced using cashew apple juice as substrate presented higher stability than the crude enzyme produced using synthetic medium, presenting the same behavior previously reported for dextranase from *L. mesenteroides* B-512F [17]. The optimum stability conditions were 25°C and pH 6.5, with good enzyme stability for 48 h. The use of crude enzyme from *L. citreum* B-742 in enzyme process, such as oligosaccharides synthesis in cashew apple juice is subject of future works.

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