# EFFECT OF STOPPING FERMENTATION OF *Leuconostoc mesenteroides* ATCC 10830 (B-512F) AT DIFFERENT GROWTH STAGES ON DEXTRAN FORMATION AND MEDIUM VISCOSITY

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## ABSTRACT

*At the cut end of deteriorated sugar cane stalks, Leuconostoc mesenteroides grows, secreting dextransucrase and forming dextran. When biocide is sprayed, bacteria will be killed but dextransucrase might still be active and forming dextran.*

*In this experiment, it was found that when fermentation was stopped (L. mesenteroides were killed) this enzyme (dextransucrase) was still able to form high concentration of dextran. The amount of dextran formed depended on the time of fermentation. It also depended on the pH and temperature during the incubation which affect the activity and the stability of dextransucrase. The higher the incubation temperature (20-30°C), the more sensitive dextransucrase activity to the pH changes (pH 4.6 – 5.4). The highest dextransucrase stability during a 20 h incubation was found at pH 5,4. The highest activity was found at 30° C for pH 5.4 while at 25°C the activity was only slightly lower than 30°C.*

***Key words:*** *dextran formation, medium viscosity, dextransucrase.*

## INTISARI

*Pada potongan batang tebu yang rusak Leuconostoc mesenteroides tumbuh, menghasilkan dextransucrase dan membentuk dekstran. Apabila biosida disemprotkan, bakteri akan terbunuh tetapi dextransukrase masih aktif membentuk dekstran.*

*Dalam penelitian ini, didapatkan bahwa ketika fermentasi dihentikan (L. mesenteroides dimatikan, ternyata enzim ini (dextransucrase) masih mampu menghasilkan dekstran dengan konsentrasi yang tinggi. Jumlah dekstran yang dihasilkan tergantung dari lama waktu fermentasi. Jumlah dekstran yang dihasilkan juga tergantung pada pH dan suhu selama inkubasi yang akan berpengaruh pada aktifitas dan stabilitas dekstransukrase. Semakin tinggi suhu inkubasi (20-30°C), semakin sensitif aktifitas dekstransukrase terhadap perubahan pH (pH 4,6-5,4). Stabilitas dekstransukrase yang paling baik selama 20 jam inkubasi adalah pada pH 5,4. Aktifitas dekstransukrase paling tinggi adalah pada 30°C untuk pH 5,4. Untuk suhu 25°C aktifitas dextransukrase hanya sedikit lebih rendah dibanding pada suhu 30°C.*

***Kata-kata kunci:*** *pembentukan dekstran, viskositas medium, dekstransukrase.*

## INTRODUCTION

Dextran has been a problem in the sugar industry for many years. Dextran is a glucan with a main chain of α(1-6) linked glucose residues and α(1-3) branched glucose units. Dextran is formed by an extracellular enzyme, dextransucrase (E.C. 2.4.1.5), produced by *Leuconostoc mesenteroides* which polymerizes sucrose into dextran.

Nowadays, this bacteria is generally considered to be the most important factor contributing to the post harvest deterioration of sugar cane. The significant losses of sucrose due to this bacteria include the conversion of sucrose into dextran, but also losses related to increasing cane juice viscosity which creates problems in further processing of cane juice in the factory[1-5].

In this study, the focus of attention was the formation of dextran by *L. mesenteroides* ATCC 10830 (B-512F) because the dextran produced by this strain has similar characteristic to the dextran isolated from deteriorated sugar cane (95% α-1,6 and 5% α-1,3)[7].

When sugar cane stalk starts to deteriorate, *Leuconostoc mesenteroides* grow at the cut end of cane stalk, secreting dextransucrase and forming dextran. When biocide is sprayed, bacteria will be killed but dextransucrase might still be active and forming dextran. This research project was aimed to study the formation of dextran when the fermentation is stopped (*L. mesenteroides* are killed) at different stages but dextransucrase was left active.

## MATERIALS AND METHODS

### Organism

*Leuconostoc mesenteroides* strain ATCC 10830 (NRRL-B512(F)) was used and obtained from the ATCC. It was maintained on All Purpose Tween Agar (APTA) (Merck) (4°C) and All Purpose Tween Broth (APTB). APTA (15 g) was dissolved in distilled water (500 ml total volume), divided into small containers and sterilized by autoclaving at 121°C for 15 min. One liter of APTB contained: peptone from casein (Merck), 12 g; yeast extract (DIFCO), 7.5 g; D(+) glucose (Riedel de Haen), 10 g ; NaCl (BDH analar), 5.0 g; trisodium

citrate dihydrate (BDH), 5.70 g; $K_2HPO_4.3H_2O$ (BDH), 6.55 g; Tween 80 (Sigma), 0.2 g; $MgSO_4.7H_2O$ (Mallinkrodt), 1.637 g; $MnCl_2.4H_2O$ (BDH), 0.22 g; $FeSO_4.7H_2O$ (BDH), 0.073 g; thiamidium dichloride (Sigma), 0.001 g. The cultures were regenerated every two months.

## INOCULUM PREPARATION

Two loops of *L. mesenteroides* were inoculated into 100 ml APTB in 250 ml erlenmeyer stopped with cotton wool and were grown overnight for 16 h (28°C, static culture in a water bath). The culture obtained was diluted using 0.9% (w/v) NaCl to an absorbance of 0.4 (900 nm) and the resulting solution was used as a seed culture. 1 ml seed culture contained approx. 5 x $10^8$ living cells. About 5.4 % (v/v) seed culture was used for inoculation of the growth medium.

### Growth medium

The LKM 100 medium used by Otts and Day[8] for dextransucrase production was slightly modified. Tween was not used and sucrose concentration was increased to 15% (w/v). The modification was done to mimic sugar cane juice composition[9].

One liter of medium contained: tryptone (Merck), 10 g; yeast extract (DIFCO), 7.9 g; $K_2HPO_4.3H_2O$ (BDH), 11.4 g and sucrose, 150 g. All media pH were adjusted to 6.9 - 7.0 using $H_3PO_4$ 51% (w/w) before autoclaving at 121°C for 15 min.

### Fermentation conditions

Fermentation conditions were selected to mimic the growth of *L. mesenteroides* ATCC 10830 (B-512F) on the cut end of the sugar cane stalk. All fermentations, were conducted at 30°C (water bath, static culture) in erlenmeyer stopped with cotton wool. Phosphoric acid was added to adjust the initial pH of medium to pH 5.7 before fermentation was started. Sodium azide (1M stock solution in distilled water) was added (10mM final concentration) to stop fermentation without affecting dextransucrase activity[10, 11].

When required, dextransucrase was initially inactivated by heating for 3 min at 90°C[12]. The treatment was equivalent to inactivation of dextransucrase by soaking the sample (either in a 10 ml Kimax test tube or 50 ml erlenmeyer) in boiling water for 5 min.

### Measurement of growth

The growth of *L. mesenteroides* ATCC 10830 (B-512F) was followed by measuring the absorbance at 900 nm.

### Dextran analysis

*Analysis of dextran using the Haze method (modified from Mochtar and Rachman)*[13].

The haze method is routinely used to determine the dextran concentration in sugar cane juice during processing in the sugar cane factory, because this method is quite fast and simple.

TCA 10% (w/v) (0.1 ml) was added to 0.5 ml sample at the appropriate dilution, followed by 1.87 ml ethanol (96% v/v). After mixing using a vortex, and 20 min stand at room temperature, the haze was read at 720 nm against a blank (medium without dextran, at the same dilution as the sample). A minimum of 4-fold dilution was needed to avoid excessive haze interference from other compounds which were present in the fermentation medium. The $2.10^6$ MW dextran standard curve was chosen to calculate the dextran concentration of the fermented sample, as it corresponded best to the molecular weight range of dextrans produced by *Leuconostoc mesenteroides* B-512F (ATCC 10830).

### Viscosity measurement

Viscosity may be defined mathematically by

$$\eta = \text{viscosity} = \frac{\tau}{\gamma} = \frac{\text{shear stress}}{\text{shear rate}} \quad \ldots . \qquad (1)$$

The fundamental unit of viscosity measurement is the "poise". A material requiring a shear stress of one dyne per square centimeter to produce a shear rate of one reciprocal second has a viscosity of one poise, or 100 centipoise[14].

The ratio of the shear stress to the shear rate (viscosity) is constant for a Newtonian fluid (see equation 1).

For a non Newtonian fluid this ratio is not constant, but varies with the applied shear rate, and is given as the apparent viscosity (equation 2)

$$\eta_{app} = \tau/\gamma \quad \ldots . \qquad (2)$$

where $\eta_{app}$ is the apparent viscosity

In this study, samples varied from non Newtonian to Newtonian liquid. In order to compare the samples viscosity, apparent viscosity had to be determined at the same shear rate.

Using the Brookfield cone plate viscometer (LVDV II) it was impossible to measure all the samples viscosity at the same shear rate (rpm) due to the equipment limitations. The high viscosity samples could only be measured at low shear rate (rpm), on the other hand low viscosity samples could only be measured at high shear rate.

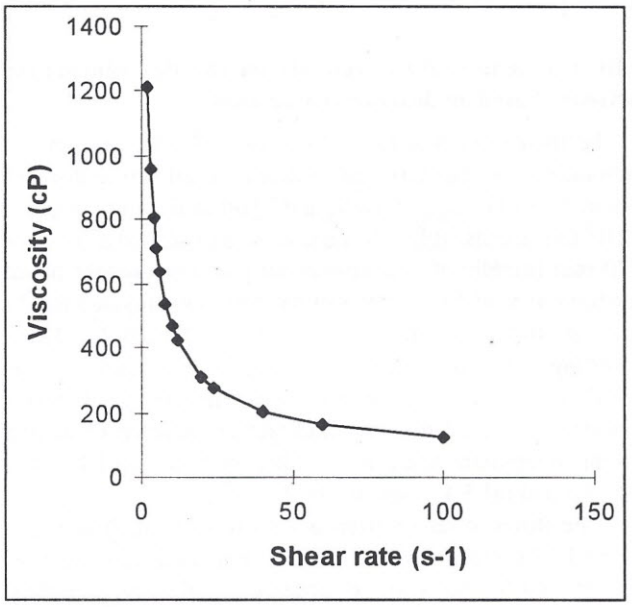
In this study the apparent viscosity was calculated at a shear rate of 10 $s^{-1}$ (temperature 25°C) based on Ostwald-de Waele equation[15].

$$\tau = K\ \gamma^n \quad \ldots . \qquad (3)$$

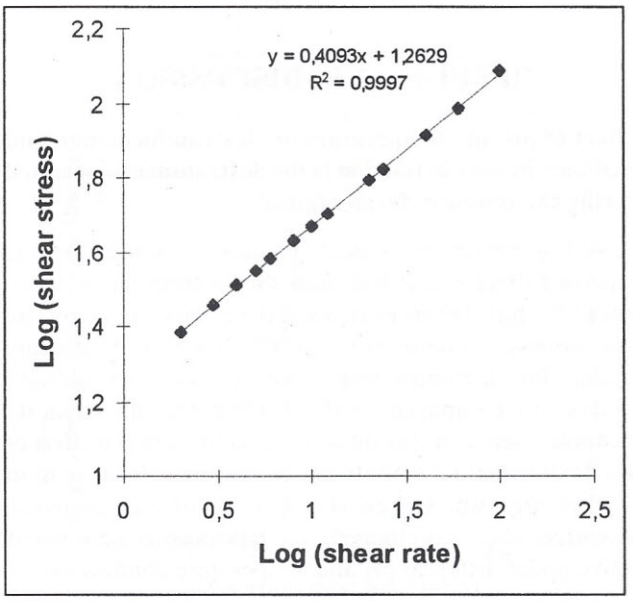
$$\log(\tau) = \log(K) + n \log(\gamma) \quad \ldots . \qquad (4)$$

K = consistency index (poise.$s^{(n-1)}$); n = power law index, $\tau$ = shear stress (dyne/$cm^2$), and $\gamma$ = shear rate ($s^{-1}$).

Figure on the *Appendix 1* shows a typical behavior of samples during viscosity measurement. It showed shear thinning behavior: the viscosity decreased with increasing shear rate. The relation between log (shear rate) and log (shear stress) was linear and could be fit into Ostwald de Waele equation (equation 4) (see Figure on the *Appendix 2*). Log($K$) and n could be determined from the linear equation. The shear stress ($\tau$) could then be calculated, assuming a shear rate ($\gamma$) 10 $s^{-1}$, using equation 4. Then, the apparent viscosity at a shear rate of 10 $s^{-1}$ was calculated using equation 2.




**Apendix 1.** Typical rheological behavior of samples (shear thinning behavior)




**Apendix 2.** The relation between log (shear rate) and log (shear stress) which fit into Ostwald de Waele equation

Viscosity was determined using Brookfield cone plate viscometer (LVDV II) at 25°C.

Two type of spindle were used in this study: CP 40 (angle, 0.8°; radius, 2.4 cm; sample size 0.5 ml, shear rate = (7.5 x RPM)) and CP 52 (angle, 3.0°; radius, 1.2 cm; sample size 0.5 ml, shear rate= (2.0 x RPM)). CP 40 was used to determine the viscosity of samples with apparent viscosity at shear rate 10 $s^{-1}$ less than 350 cP, and CP 52 for samples with apparent viscosity over 350 cP (calculated at the same shear rate).

During the viscosity measurement, the shear rate (rpm) was varied over the range of available rpm, and measurement was started from low shear rate to high shear rate.

When the sample contained bacteria cells, it had to be centrifuged. Centrifugation was done using a microfuge (room temperature), at 20,000 g for 20 min. Centrifugation at room temperature was chosen in order to minimize precipitation of high molecular weight dextran together with bacteria cells. The complete supernatant was collected and vortexed for around 15 second.

All samples were stored at -18°C before viscosity analysis. Samples were thawed and heated until 25°C in a water bath set at 30°C. Sample was vortexed, and temperature was checked again before sample was taken for viscosity measurement. The purpose of adjusting the temperature to 25°C before the sample was taken for viscosity measurement was to reduce the time for the sample to reach an equilibrium temperature before the viscosity measurement started. A temperated sample was placed into the cone plate viscometer cup and kept for 2 min before viscosity measurement started. Sample preparation procedure has been tested before (data not shown).

**Effect of incubation temperature on dextran formation and medium viscosity in relation to the dextransucrase secreted during the course of fermentation**

Experiment was conducted in 50ml-erlenmeyers. Each erlenmeyer contained 30 ml medium and 4 ml distilled water. Erlenmeyers were sterilized at 121°C for 15 min. $H_3PO_4$ 17% (w/v) 0.6 ml was added to adjust the initial pH of the medium to 5.7. Two milliliters seed culture were added and the erlenmeyer was put on ice before incubation started. Twenty one erlenmeyers were incubated for different time, which will represent different stages during fermentation at 30°C. The fermentation time were; 0, 120, 180, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 320, 340, 360, 380, 420, 480 and 660 min. Fermentation was stopped by adding sodium azide, but dextransucrase was not inactivated.

When the sampling points were very close to each other (10 min difference), it was impossible to take a sample one by one and centrifugated for 20 min. Hence, the starts of fermentation were set up such that at the end of fermentation four samples could be harvested at the same time. Under these conditions, the medium wich contained bacteria was stored on ice for a maximum of 5 h. It has been tested that the medium

containing bacteria could be stored on ice until 8 h without any changes in it's performance.

Approximately 10 ml aliquot of fermented medium was taken from each erlenmeyer into a 10 ml Kimax test tube and heat-treated to inactivate dextransucrase (5 min, boiling water). This sample was used for viscosity analysis and pH determination.

The remaining of the fermented medium (approximately 25 ml), with active dextransucrase was centrifuged at 20,400 g, 30°C for 20 min using Beckman J2-21M/E centrifuge and used for further enzyme studies.

At some stages during fermentation, after centrifugation of the sample, a loose milky layer was observed on top of the pellet, mainly during the exponential phase of growth. This layer, thought to be dextrans, was included with the supernatant. This loose milky layer disappeared when the bacteria reached a stationary phase, where the hydrolyzing enzyme was secreeted into the medium. It was hypothesized that the presence of hydrolizing enzyme could hydrolyze the high molecular weight dextran which formed the milky layer.

The supernatant containing the active dextransucrase was divided into two equal aliquots. The first aliquot was divided into 3 vials (approx. 4 ml per vial) and incubated at three different temperature (20, 25, 30°C) for 20 h. To the second aliquot, sucrose was added (1 g per 10 ml supernatant (sucrose concentration approx. 10% w/v)), then the mixture was divided evenly into 3 vials and incubated as previously described. After 20 h incubation, the dextransucrase was inactivated.

**Effect of adjusting the fermented medium pH to 5.0 on dextransucrase activity during the course of fermentation**

Experiment was conducted in 50 ml-erlenmeyers. Each erlenmeyer was filled with 15 ml medium, 1.5 ml distilled water, and sterilized at 121°C for 15 min. The pH was adjusted by the addition of $H_3PO_4$ 17% (w/v) 0.28 ml. 1 ml seed culture containing approx. 5 x $10^8$ living cells was added before fermentation started.

The erlenmeyers were incubated for different times which represented different stages during fermentation at 30°C. Samples were taken at 0, 120, 200, 230, 250, 260, 280, 290, 300, 320, 360, 400, 460, 625 min. Fermentation was stopped by adding sodium azide, but dextransucrase was not inactivated. The fermentation was set-up as previously described.

One ml of sample was taken for the dextransucrase activity analysis and another 1 ml was used for growth determination. The remaining fermented medium was divided into two aliquots. An aliquot (5 ml) was used for pH measurement (after heat treatment). Another aliquot (10 ml) with active dextransucrase was centrifuged for 20 min (20 400 g, at 30°C) using a Beckman J2-21M/E centrifuge. The pellet was discarded. 0.5 ml of 2 M acetate buffer (pH 5.0)* was added to 4.5 ml supernatant. [1]Both solutions were incubated at 30°C for 20 h. At the end of the incubation, the dextransucrase was inactivated and the dextran content was determined.

When the pH of the fermented medium dropped under 4.6, addition of 1M KOH was added to adjust pH to 5.0. 1 M KOH was not added directly into fermented medium. The required amount was first added to 2 M acetate buffer pH 5, then the mixture was added to the fermented medium. The required amount was determined beforehand using medium A which has been adjusted to the corresponding pH using lactic acid. Then the solution was used to determine how much additional 1M KOH was needed to adjust pH to 5.0.

**Effect of temperature and pH on the dextransucrase activity (based on dextran synthesized)**

Fermentation was carried out in a 500 ml erlenmeyer containing 300 ml sterilized medium, 30 ml sterile distilled water, 5.6 ml $H_3PO_4$ 17% (w/v) and 20 ml seed culture (approx. $5x10^8$ living cells / ml). The mixture was incubated at 30°C for 270 min (middle of the exponential phase of growth; pH of medium around 5.0). The sample was centrifugated for 20 min, 20,400 g (set at 30°C) using a Beckman J2-21M/E centrifuge. The supernatant containing the dextransucrase was filled into three series of 50ml-erlenmeyers. Each series consisted of five erlenmeyers, and each erlenmeryer contained 18 ml supernatant and 2 ml 2 M acetate buffer (pH 4.6, 4.8, 5.0, 5.2 and pH 5.4, respectively (NaOH)).

The three series of erlenmeyers were incubated at 20, 25 and 30°C, respectively. At the indicated time (0, 3, 8, 12, 16, 20 h), an aliquot (approx. 3 ml) was sampled, dextransucrase was inactivated and the dextran content was determined.

The experiment was done in duplicate started from fermentation.

## RESULT AND DISCUSSION

**Effect of pH and temperature on dextran formation and medium viscosity in relation to the dextransucrase secreted during the course of fermentation**

A low buffering capacity medium was used in this experiment because it had been shown from the previous work[16] that fermentation using this medium by *Leuconostoc mesenteroides* ATCC 10830 (B-512F) only produce low dextransucrase activity and low concentration of dextran compared to the high buffering capacity medium. Hence, it was interesting to find out the effect of low dextransucrase activity produced during fermentation of this medium (when the growth of *Leuconostoc mesenteroides* was stopped, and dextransucrase was left active under different pH and temperature conditions).

*Figure* 1(a), *Figure* 1(b) and *Figure* 1(c) illustrate the growth curve of *L. mesenteroides*, medium pH and medium viscosity profiles during fermentation at 30°C, respectively.

*) (100 ml 2 M acetate buffer (pH 5) was prepared from the mixture of 29.6 ml 2M actic acid and 70.4 ml 2 M sodium acetate).

The exponential phase of growth started around 230 min fermentation, and the growth stopped after 300-350 min fermentation when the pH reached approx. 4.5. During that period medium viscosity increased from approx. 1 cP to 6 cP (at shear rate 10 $s^{-1}$) and after 400 min fermentation it decreased due to the effect of hydrolyzation (*Figure* 1c)[16].

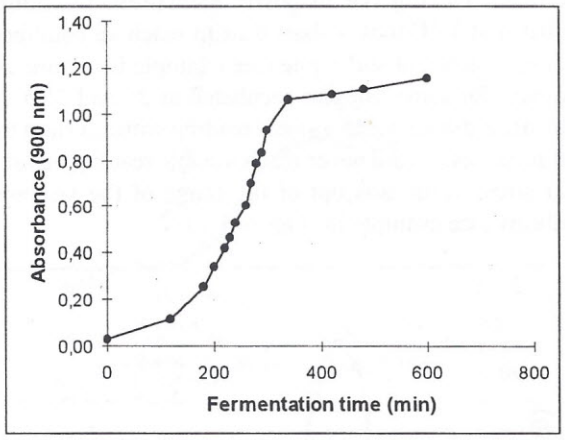



**Figure 1(a).** Growth curve of *L.mesenteroides* ATCC 10830 (B-512F) during fermentation

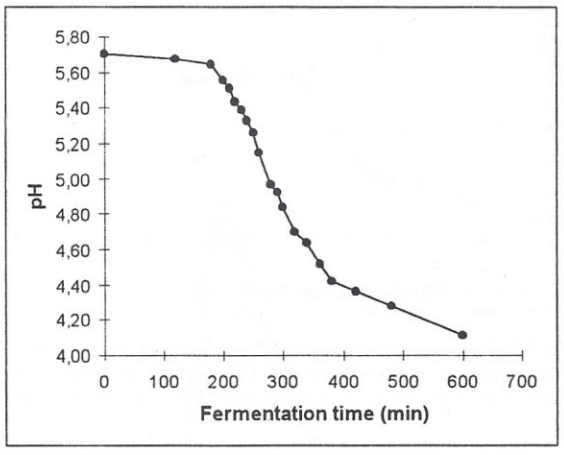



**Figure 1(b).** Evolution of medium pH during fermentation by *L.mesenteroides* ATCC 10830 (B-512F)

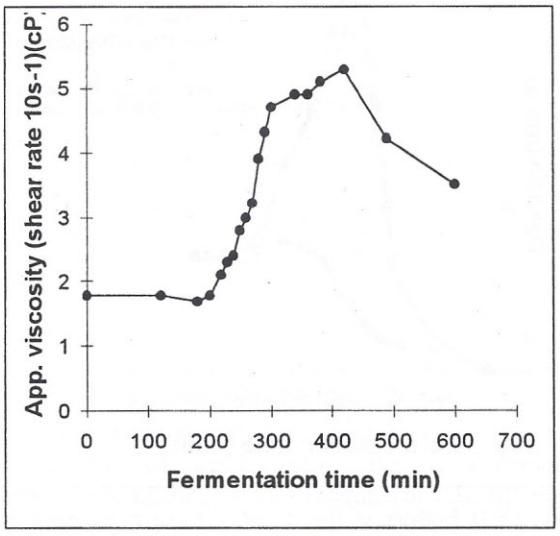



**Figure 1(b).** Evolution of medium pH during fermentation by *L.mesenteroides* ATCC 10830 (B-512F)

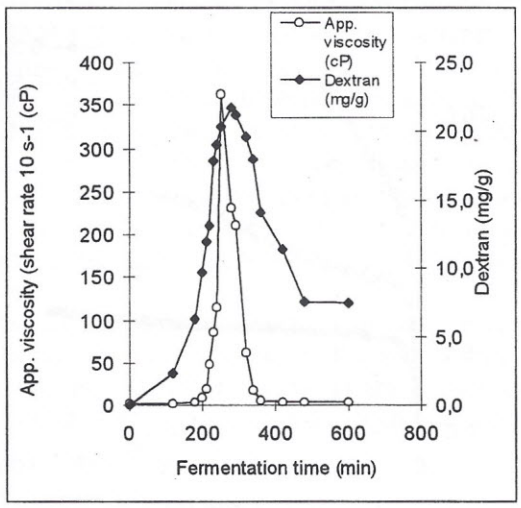



**Figure 1(d).** Evolution of dextran content and viscosity of meidum during the incubation at 30 C from different stages during fermentation by *L.mesenteroides* ATCC 10830 (B-512F)

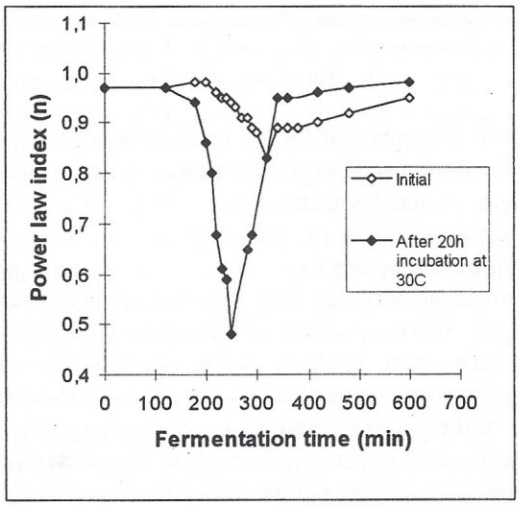



**Figure 1(e).** Evolution of the power law index (n) of medium during incubation at 30 C from different stages during fermentation by *L.mesenteroides* ATCC 10830 (B-512F)

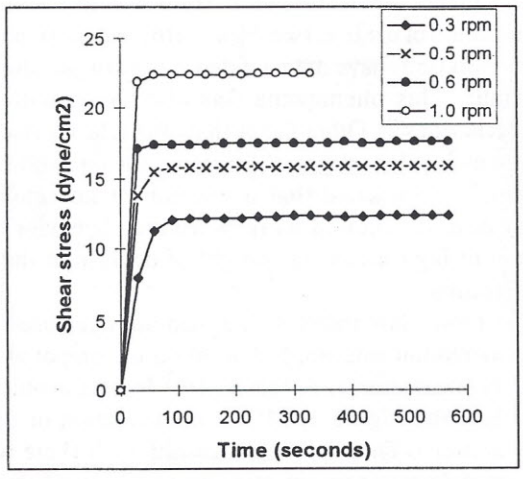



**Figure 1(f).** Evolution of shear stress over time at different shear rates during rheology measurement of medium after 260 min fermentation by *L.mesenteroides* ATCC 10830 (B-512F), followed by 20 h incubation at 30 C

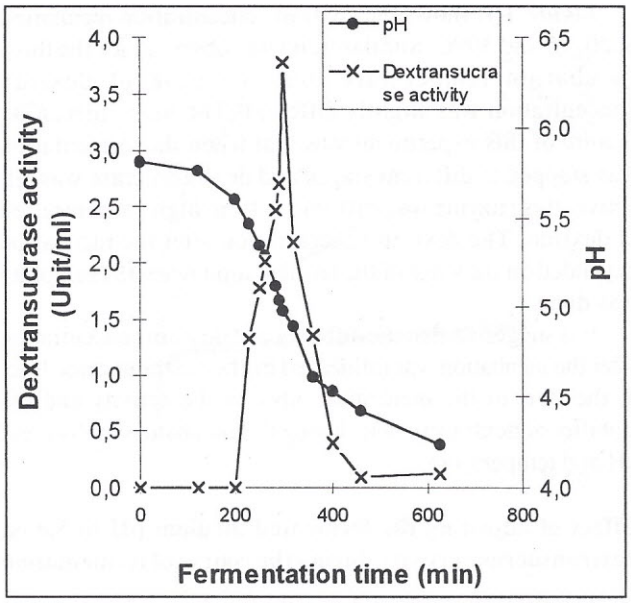



**Figure 2(b).** Evolution of dextransucrase adtivity and medium pH during fermentation by *L.mesenteroides* ATCC 10830 (B-512F)

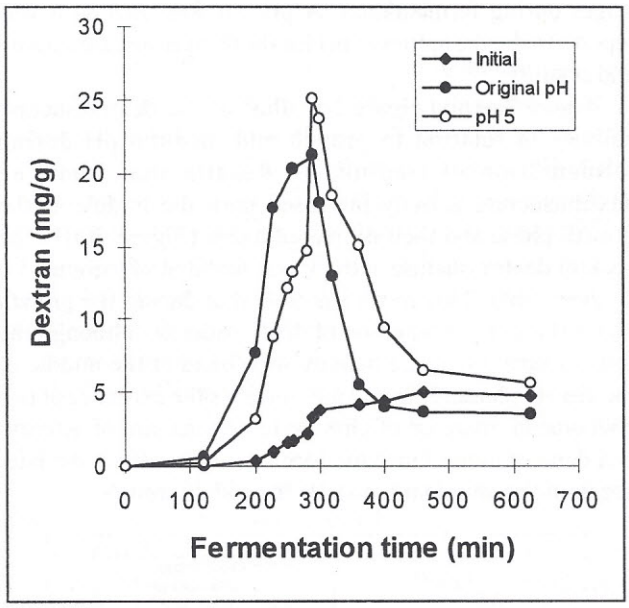



**Figure 2(c).** Evolution of dextran content after 20 h incubation at 30 C from different stages during fermentation by *L.mesenteroides* ATCC 10830 (B-512F)

*Figure* 2(c) shows that when the pH of medium was below 5, although the pH of medium was eventually brought back to 5 (optimum of dextransucrase activity) the enzyme activity could not be recovered. Indeed profile of the dextran of the fermented samples after the incubation, in absence of bacteria was very similar to the profile of dextran concentration of the corresponding samples after the incubation when pH was adjusted to 5 before incubation started. Looking at the increasing part of the curve, the dextran concentration after 20 h incubation of the fermented medium (pH varied between 5.0-5.4) was higher compared with the dextran concentration after incubation of the medium which pH has been adjusted to 5.0 before incubation started. This indicated that the activity and stability of the dextransucrase was higher when pH of medium was between 5.0-5.4.

**Effect of temperature and pH on the dextransucrase activity (based on dextran synthesized)**

The effect of different temperature and pH of medium during the the incubation on dextran formation (see *Figure* 3(a), *Figure* 3(b) and *Figure* 3(c)) was further investigated. This experiment showed clearly that the dextransucrase was not very stable at pH 4.6 and 30°C, but still quite stable at pH 4.6, at 20 and 25°C (*Figure* 3(d)). Between pH 5.0–5.4, the dextransucrase was quite stable during the incubation at 20, 25 and 30°C, although the rate of dextran formation was different. The higher temperature the more sensitive was the dextransucrase to the pH changes.

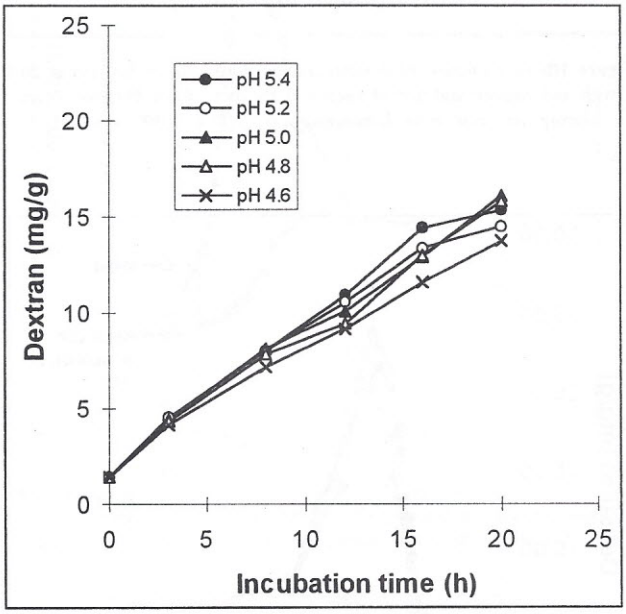



**Figure 3(a).** Evolution of dextran content during the incubation at 20 C in different pH

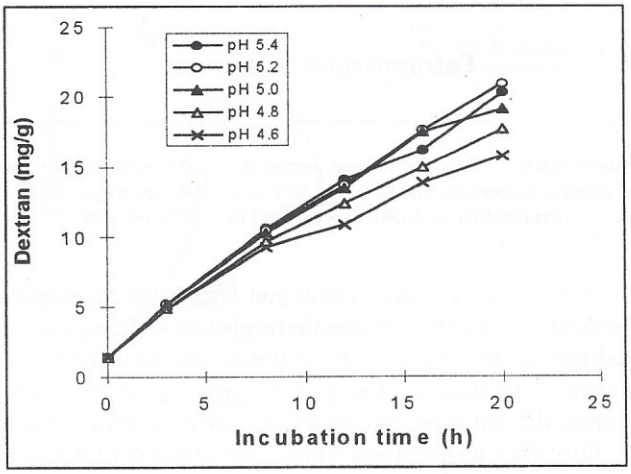



**Figure 3(b).** Evolution of dextran content during the incubation at 25 C in different pH

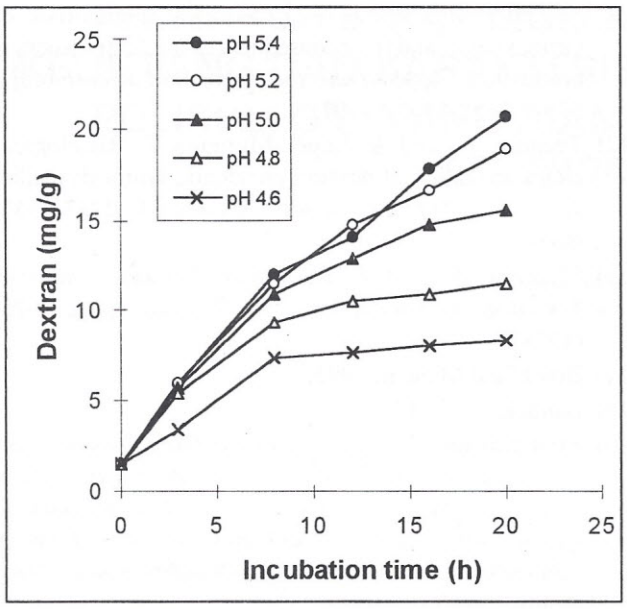



**Figure 3(c).** Evolution of dextran content during the incubation at 30 C in different pH

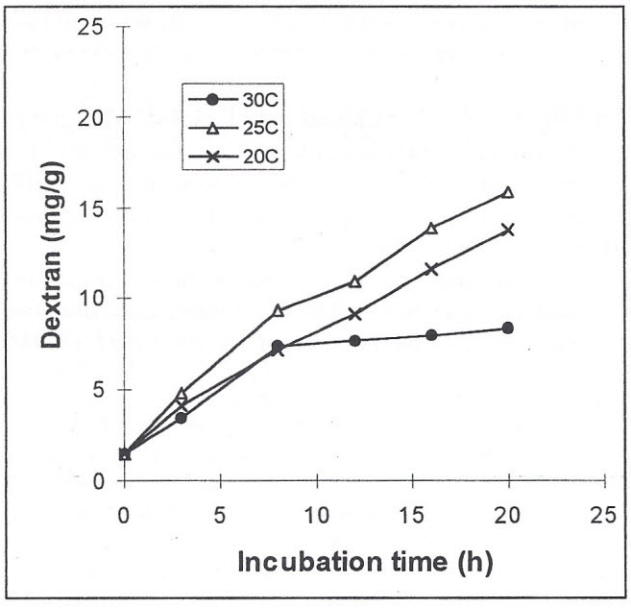



**Figure 3(d).** Evolution of dextran content during the incubation at pH 4.6 and three different temeprature

It is interesting to note that in this experiment, 30°C is the critical temperature for the stability of the dextransucrase in relation with pH sensitivity. Kaboli and Reilly[18] also reported that the higher the temperature the faster the enzyme decay (temperature test between 25 - 35°C). This explained why dextran concentration after the incubation at different temperature showed slightly different peaks (see *Figure* 1(i)).

Other factor that may affect the activity and the stability of dextransucrase was the dextran concentration before the incubation started (in this experiment varied from 0 to around 6 mg/g). Kim and Robyt[21] reported that the native dextran formed *in situ* by dextransucrase up to 3 mg/ml concentration increased enzyme activity, although it was not known what mechanism involved in that phenomena.

Based on the above results of experiments, it was found that when *L. mesenteroides* ATCC 10830 (B-512F) were killed at different stages during fermentation without affected dextransucrase activity, it showed that this enzyme was still able to form dextran at various concentration which depended on the stage of fermentation.

It is suggested that the right biocide for the field application has to be carefully chosen and the time when it will be sprayed on the cane has to be carefully considered. Some biocides may be able to kill bacteria without having dextransucrase inhibitor properties. If the biocide with low dextransucrase inhibitor activity is sprayed on the fresh sugar cane stalk, the reduction of the dextran after several days in storage might be significantly different compared with control. On the contrary when this biocide is sprayed on the slightly deteriorated sugar cane, the increase on dextran content compared with the control after further storage might be happened. This suggestion is based on the experimental results that when fermentation of *Leuconostoc mesenteroides* is stopped in the middle of the growth phase, dextransucrase is still able to form dextran where the dextran content became even higher than normal fermentation after the incubation. However, this suggestion never been tried on the field test before.

The best biocide should have a good properties as an anti-bacteria and also as dextransucrase inhibitor with reasonable price to minimize dextran formation which relate with viscosity problem in sugar industry.

## CONCLUSIONS

When the fermentation was stopped (*L. mesenteroides* are killed) and the dextran sucrase was left active, it was demostrated that this enzyme was still able to form high concentration of dextran. The amount of dextran formed with time depended on the dextransucrase activity at the point when fermentation was stopped. It also depended on the pH and temperature during the incubation, which affected the activity and stability of the dextransucrase: the higher the temperature during the incubation (20–30°C), the more sensitive dextransucrase activity to the pH changes (pH 4.6 - 5.4). The highest dextransucrase stability during the incubation is found at pH 5.4. The optimum pH for the highest activity at 30°C was found at pH 5.4. At 25°C the activity was only slightly lower than 30°C.

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