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Research article

A comparison of two enzymatic methods of clinical dextran production

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Abstract: *The aim of this study was to evaluate and compare of the two enzymatic methods of clinical dextran production were compared. The reactions were performed at 30°C and pH 5.4 in solutions containing different amounts of sucrose, using dextransucrase (DS, in the presence of dextranase (D) (method 1) or acceptor dextrans (method 2). The activity of Leuconostoc mesenteroides L dextransucrase (DS), which converts sucrose to dextran, was 0.4 U ml^{-1} in both the methods. As much as 53-56% of clinical dextran fractions were obtained for 28 h from 10% sucrose solutions, which contained 1.5% or 2.5% acceptor dextrans with molecular mass of 10 and 15 kDa, respectively. Approximately 50% of these fractions was obtained (also in 28 h) from 10% sucrose solutions by using 0.004 U ml^{-1} of DN, added to reaction mixtures 5 h later than Our experiments indicate that the clinical dextran can be efficiently produced by using both the compared methods, which employ either acceptor dextrans with definite molecular mass, or the dextranase. Because consumption of the latter enzyme is rather small, and it is easily available, thus this method should be attractive for clinical dextran manufacturers.*

Keywords: *dextransucrase, dextranase; acceptor dextrans, clinical dextran.*

Introduction

Dextrans are extracellular polysaccharides produced by *Lactobacillaceae* bacteria of the genera *Leuconostoc* and *Streptococcus* when cultured in media containing sucrose as a sole carbon source. Their main chain is built of α-1,6-linked D-glucopyranose residues, and the branchings are attached *via* α-1,3, α-1,4, and α-1,2-bonds. The linear dextran with a suitable molecular mass has been used in medicine as blood plasma replacer (dextran 70 with an average molecular mass of 70 kDa), and as a factor regulating the rate of blood flow (dextran 40 with an average molecular mass of 40 kDa) [1]. The first and simplest, however also the most expensive method of dextran production is

a multi-step process, based on synthesis of high molecular weight, native dextran from sucrose either by *Leuconostoc mesenteroides* cells or by the purified dextransucrase of this bacterium, the polymer isolation, its partial controlled enzymatic or acid hydrolysis, and precipitation of fractions with different molecular weights with organic solvents, such as ethanol and methanol [2].

To simplify dextran production, some other methods were proposed, including enzymatic synthesis, which involved sugar acceptors. In 1950s it was observed that apart the high molecular weight dextran also oligosaccharides were synthesized in the presence of some sugars in reaction mixtures, which contained sucrose and dextransucrase [3]. The most efficient glucosyl residues acceptors were found to be maltose, isomaltose and low molecular weight dextrans.

To synthesize dextran with a definite molecular mass, a multi-step synthesis catalyzed by the purified *L. mesenteroides* B-512 F dextransucrase was used by Paul et al. [4] and Remaud et al. [5]. In the first step, the process was conducted in 12% sucrose (S) solution enriched with maltose (M), at the S/M ratio ranging from 2.1 to 6.5 by weight. Molecular masses of the oligosaccharides formed due to acceptor reactions were linearly proportional to the S/M ratio. These oligosaccharides played the role of acceptors in the second step of synthesis, and molecular mass of reaction products was dependent not only on the S/A ratio, but also on molecular mass of the acceptors. Dextran with molecular mass of approximately 100 kDa was produced in the next process steps, which involved low molecular weight dextrans derived from the former stages. This method has not been commercialized because of economic reasons.

In 1960s, Novak and Witt [6] patented the clinical dextran production method, which employed *Aspergillus wentii* dextranase. The *A. wentii* culture broth filtrate was mixed with sucrose solution and inoculated with *L. mesenteroides*. Dextran with molecular mass of 50-100 kDa (30% yield of the process) was obtained after 18 h culture.

In 1990s, Kim and Day [7-9] invented a single-step method of dextran production, which involved the mixed culture of the bacterium *L. mesenteroides* B-512F and the yeast *Lipomyces starkeyi* ATCC 74054. Dextransucrase, secreted by the bacterium, converted sucrose to dextran and fructose, and the yeast dextranase partially hydrolyzed the newly formed polymer. Under optimized culture conditions (pH 5.2, 28°C), 73-85% conversion of sucrose, concentration of which was kept at 30%, was achieved, and the clinical dextran constituted 93% of all polysaccharides formed in this process. The only drawback of the latter method was a high protein content in the product.

Moulis et al. [10] proposed a new method for obtaining dextrans of controlled molecular weight. The concept based on the use of only one enzyme, on the use of engineered *Leuconostoc mesenteroides* NRRL B-512B dextransucrase variant. Under optimum reaction conditions, the polymer o molecular weight of 40 kDa was obtained in 75% yield.

Chen et. al [11] developed a new separation method to control the molecular weight distribution. The quality of clinical dextran was improved by the use electric ultrafiltration in combination with solvent crystallization.

Results of Kim and Day [7-9] provided an incentive to our studies on application of partially purified dextransucrase and dextranase for production of dextran with defined molecular mass.

Experimental

Materials

Acceptor dextrans T-15, T-10, T-5 and T-3 with average molecular mass of 15, 10, 5 and 3 kDa, respectively, were purchased from pharmaceutical company KZF "Polfa" (Poland).

The enzymes, dextransucrase by *L. mesenteroides* L and dextranase by *Penicillium funiculosum* 72, were synthesized in our Institute. The strain of *L. mesenteroides* L was purchased from KZF Polfa (Kutno, Poland), where it was employed for clinical dextran production. *Penicillium funiculosum* 72 was from pure culture collection of our Institute.

Methods

Analytical methods

Dextransucrase activity was determined by measuring the initial rate of fructose production using the alkaline 3,5-dinitrosalicylic acid (DNS) solution [12]. This method could be used for dextransucrases activity assay, because glucose was not detected in reaction mixtures. It provided evidence that dextransucrase preparation contained neither invertase nor levansucrase. The reaction conditions were as follows: temperature of 25°C, pH 5.4 (0.05 M sodium acetate buffer), 0.02 g l^{-1} $CaCl_2$ and 75 g l^{-1} sucrose.

One unit of dextransucrase activity denotes 1 micromol of fructose liberated per 1 min under the above reaction conditions.

Dextranase activity assay was based on an increase in concentration of reducing sugars released from dextran T-110 (molecular mass of 110 kDa, 16.7 g l^{-1}) dissolved in 0.05 M sodium acetate buffer solution, pH 5.4. The reducing sugars were quantified according to Somogyi-Nelson [13-14].

One dextranase activity unit denotes 1 micromole of isomaltose released from dextran T-110 in 1 minute at 25°C and pH 5.4.

Dextran synthesis

Dextran synthesis was carried out at 25°C and pH 5.4 in 10% (w/v) sucrose solutions, which contained dextransucrase and either low molecular weight acceptor dextran (the reference method 2), or dextranase (authors' method 1). In the latter method, 15 and 20% sucrose solutions (initial concentrations or adjusted during the process) were also applied.

Studies on dextran homogeneity

Dextran derived by using both the methods was precipitated with a double ethanol volume, separated from the supernatant, dissolved in deionized water, and fractionated with successive ethanol concentrations of 38, 40, 42, 44, 46, 48, 50 and 60%. At each step, the mixture of dextran and ethanol was incubated at 25°C for 12 h, the precipitate was separated, and the supernatant was treated with the next portion of ethanol. The precipitated fractions were dissolved in deionized water, and the solution viscosity, and sugar concentration (by the gravimetric method) were assayed. Based on these results, the intrinsic viscosity, which is interrelated with the average molecular mass of the polymer, was computed according to the Wolff's [15] equation:

$$\eta_i = 2{,}03 \cdot 10^{-3} \cdot Mr^{0{,}431}$$

where: η_I – intrinsic viscosity, Mr – an average molecular mass.

Results and discussion

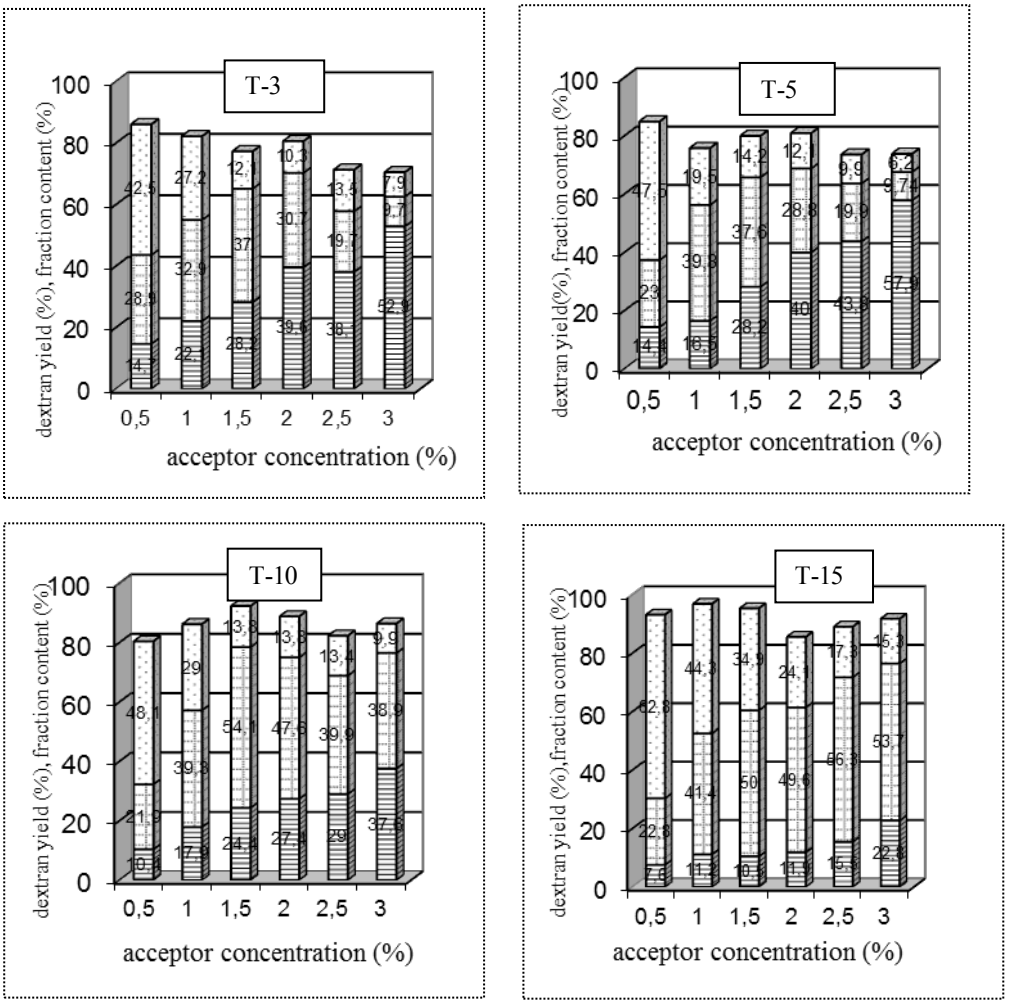
Industrially the lower molecular weight dextrans are obtained by partial acid hydrolysis of crude fermentation product (native dextran) and fractionation. However, the yields are low (10 to 12%), due to losses during the last reaction step [16]. The enzymatic method seemed to have potential for replacing the acid hydrolysis for the production of dextran polymers of controlled average number molecular weight sizes.

Presented studies were aimed at comparison of productivities of two different methods of clinical dextran synthesis. The polymer was derived from sucrose solutions by enzymatic synthesis, which involved either acceptor dextrans (to terminate the growth of dextran chain) or dextranase (to partially split the newly synthesized polymer).

Enzymatic dextran synthesis by using acceptor dextrans

Our previous studies revealed that the complete sucrose conversion in its 10% solutions, which contained 0.4 U ml^{-1} of dextranucrase lasted for 28 h [data not published]. Therefore we used these conditions in our studies on an effect of concentration (from 0.5 to 3%) and molecular mass (from 3 to 15 kDa) of the acceptor dextrans on the overall dextran productivity and its fractions percentage. The results are presented in Fig.1. An increase in concentration of T-3 and T-5 dextrans resulted in a rise in lower molecular weight fractions of reaction products. The largest amounts of clinical dextran were detected in reaction mixtures, which contained 1.5% T-3 dextran (37% of clinical fractions), and 1% T-5 dextran (40% of clinical polymer).

In the presence of acceptor dextrans with molecular mass of 10 and 15 kDa, the percentages of low molecular weight fractions of products were lessened. The high molecular weight polymer dominated when the content of these acceptors was reduced, whereas their higher content favored the clinical fractions formation.



– fractions with molecular mass > 84 700
– clinical fractions (28 900-84 700)
– fractions with molecular mass < 28 900

Figure 1. Patterns of dextran fractions (the average of triplicate samples) produced from 10% sucrose and 4 different acceptor dextrans (concentration range 0.5-3.0%) in 28 h reaction catalyzed by the dextransucrase (0.4 U ml^{-1})

The highest clinical dextran concentrations of approximately 54 and 56% were achieved in reaction mixtures containing 1.5 and 2.5% acceptor dextrans with molecular mass of 10 and 15 kDa, respectively. This results from the acceptor

reaction mechanism, due to which dextran chain associated with enzyme is transferred to the acceptor molecule [17]. Therefore the products formed when the acceptor molecule is smaller (e.g. 3 kDa) have lower molecular mass as compared to products derived by reaction with higher molecular weight acceptor (e.g. 10 or 15 kDa). A growth in acceptor concentration contributes to a rise in acceptor reaction products, and therefore low molecular weight products dominated in reaction mixtures containing 3% T-3 and T-5 dextrans, whereas their content in the presence of T-10 and T-15 acceptor dextrans was lower.
To control the molecular weight of clinical grade dextran, Lee et al. [18] added to the medium as primer dextran with a molecular weight of 10 (T-10) and 40 kDa (T-40). They demonstrated that molecular weight dextran decreased with increasing T-10 concentration, whereas the clinical dextran fraction increased continuously. When T-40 added as a primer, the high molecular fraction increased with a low yield of the clinical dextran fraction. Our research also indicated that dextran T-10 is preferred as a primer for the clinical dextran production.

Dextranase-controlled enzymatic synthesis of dextran

Molecular mass of newly synthesized dextran depends on dextranase activity in reaction mixture. Kim and Day [7-9] regulated dextran size by using selected *L. mesenteroides* (dextransucrase producer) and *Lipomyces starkeyi* (dextranase producer) cell density. In our studies, dextran synthesis was carried out in 10, 15 and 20% sucrose solutions, which contained the previously selected dextransucrase activity (0.4 U ml^{-1}), and various dextranase amounts. The latter enzyme was added to reaction mixtures immediately or at 5 h intervals from the start of dextransucrase-catalyzed reaction. The process was conducted at 25°C, and reaction products were harvested and fractionated after selected periods of time.

Synthesis in 10 % sucrose solutions

Dextran synthesis in 10% sucrose solutions was carried out for 28 h. The dextranase was added at the beginning of the process or 5 or 10 h later than dextransucrase. The results, i.e. the total dextran yield, and contents of high molecular weight, clinical and low molecular weight fractions are depicted in Fig. 2.

When the dextranase concentration was small (0.002 U ml^{-1}), only its presence in the reaction mixture from the beginning of the process provided satisfactory clinical dextran concentration (41%). When the enzyme was added later, the high molecular weight fractions dominated the products.

The most satisfactory results (50% of clinical dextran, 85% process yield) were achieved when the dextranase concentration was 0.004 U ml^{-1} and it was added after 5h of dextransucrase-catalyzed reaction.

The presence of 0.006 U ml^{-1} dextranase activity in the reaction mixture from the very beginning of the process entailed the highly advanced dextran degradation. Its total productivity dropped to approximately 62%, and the clinical and low molecular weight fractions constituted 20.4 and 42.3% of the polymer,

respectively. When the enzyme was added after 5h of the process, the high molecular weight fractions were also undetectable, and the clinical and low molecular weight ones comprised approximately 43.5 and 31% of all products, respectively. Further 5 h delay in the dextranase addition (i.e. after 10 h from the start of dextran synthesis) had a minor impact on final pattern of reaction products.

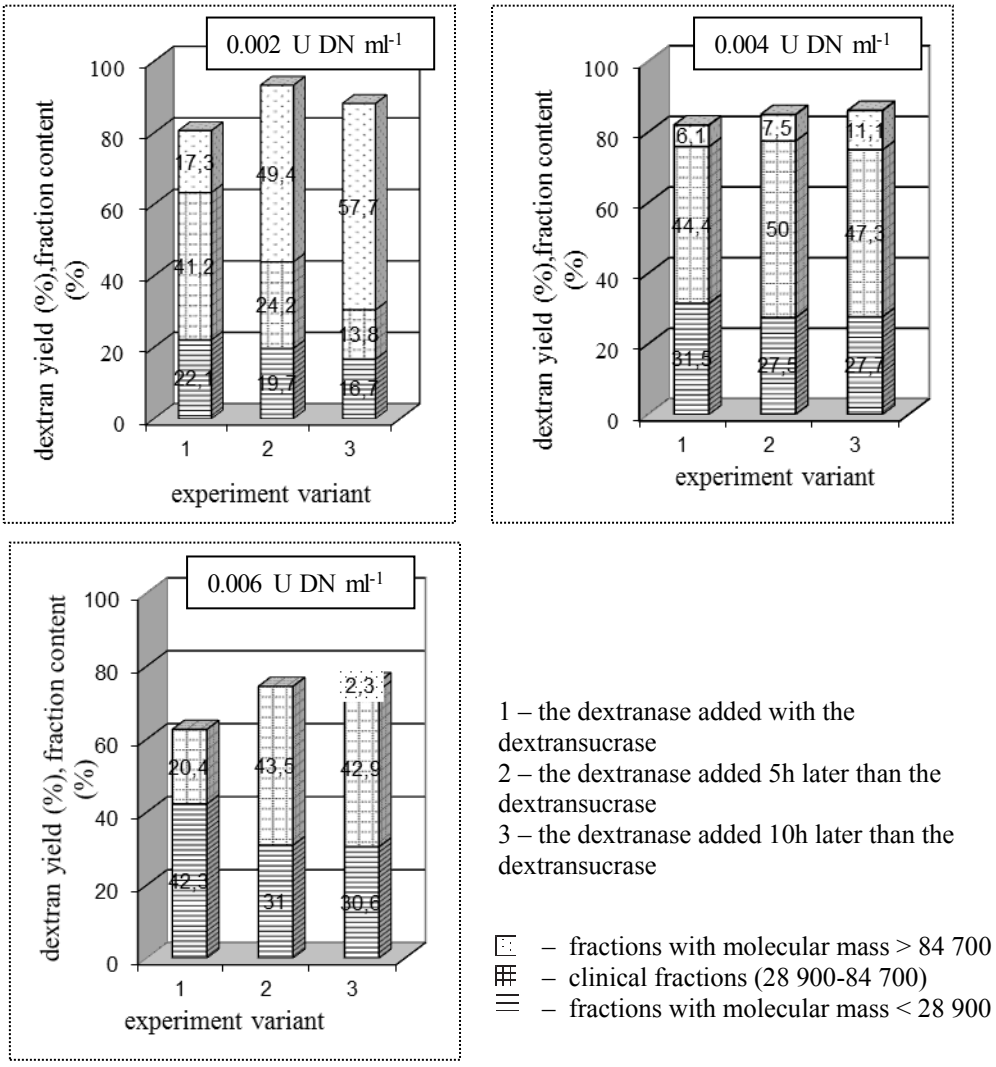
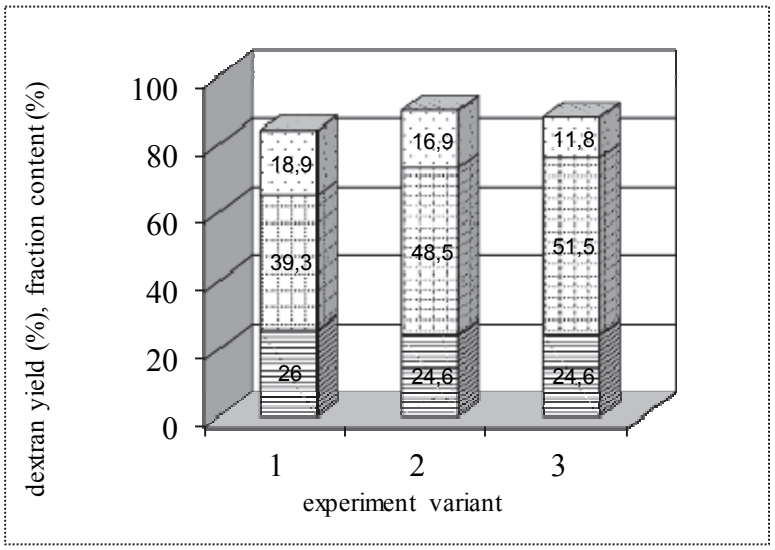


Figure 2. Percentage of dextran fractions (the average of triplicate samples) produced from 10% sucrose in 28 h reaction catalyzed by the dextransucrase coupled with the dextranase (3 different concentrations)

15 and 20% sucrose solutions were either prepared prior to the process or the sucrose concentration was gradually adjusted to these values during the process. The activity of DS was 0.4 U ml^{-1}, DN activity – 0.004 U ml^{-1}. To achieve the complete sucrose depletion, the reaction times were prolonged to 36 and 48 h, respectively. Like in the aforementioned process, the dextranase was added to the reaction mixture either at the start of dextran synthesis or 5 or 10 h later. The highest percentage of clinical dextran was found in these reaction mixtures, into which the dextranase was added after 5 h, as prove the results collected in Fig. 3 and 4.

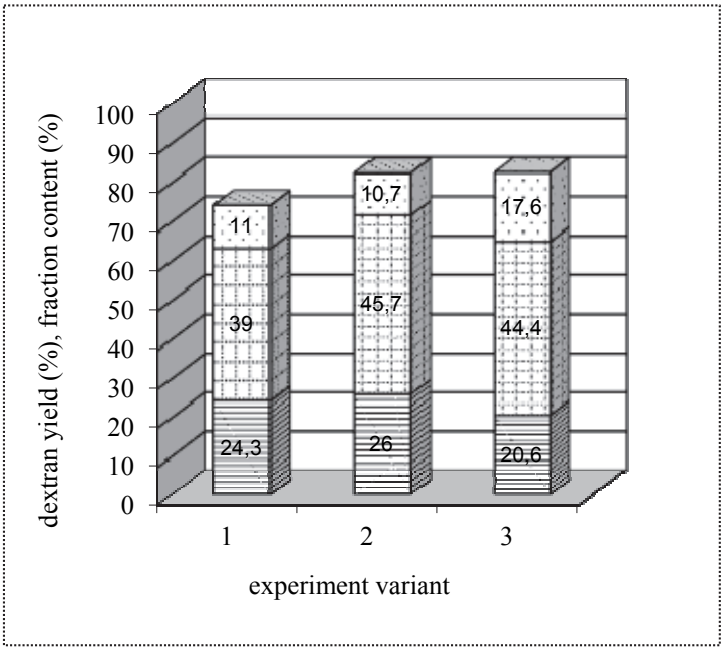


– fractions with molecular mass > 84 700,
– clinical fractions (28 900-84 700),
– fractions with molecular mass < 28 900

1 the initial sucrose concentration of 15%
2 the initial sucrose concentration of 5%, further increased to 10% after 5h, and to 15% after the next 5 h
3 the initial sucrose concentration of 10%, further increased to 15% after 10 h

Figure 3. Percentage of dextran fractions (the average of triplicate samples) produced from 15% sucrose (the initial concentration or adjusted during the process) in 36h reaction catalyzed by the dextransucrase (0.4 U ml^{-1}) and dextranase (0.004 U ml^{-1}), added 5h later than the first enzyme

The overall process productivity, achieved in 15% sucrose solutions, was approximately 84%, and the clinical fractions percentage was 39.3% (1 variant, Fig. 3). The gradual increase in sucrose concentration from 5%, to 10% after 5 h, and 15% after 10h (2 variant, Fig. 3) improved the total dextran yield to approximately 90% (the clinical fractions comprised 48.5% of the synthesized dextran). When the starting sucrose concentration was 10%, and it was risen to 15% after 10 h, the total dextran yield was 88%, and the clinical fractions constituted 51.5% of reaction products.



– fractions with molecular mass > 84 700,
– clinical fractions (28 900-84 700),
– fractions with molecular mass < 28 900

1 the initial sucrose concentration of 20%;
2 the initial sucrose concentration of 10% , further increased to 15% after 5 h, and to 20% after the next 5 h;
3 the initial sucrose concentration of 10%, further

Figure 4. Pattern of dextran fractions increased to 20% after 24 h produced from 20% sucrose (the initial concentration or achieved by sucrose supplementing) in 48h reaction catalyzed by the dextransucrase (0.4 U ml^{-1}) and the dextranase (0.004 U ml^{-1}), added 5h later than the first enzyme (the average of triplicate samples)

The total dextran yield in 20% sucrose solutions (the initial concentration) was approximately 74%, and the clinical fractions percentage was 39%. The gradual increase in sucrose concentration from 10 to 15% after 5h, and from 15 to 20% after 10 h enhanced the dextran productivity to 82%, and the clinical fractions content rose to approximately 46% (2 variant, Fig. 4). Sucrose concentration growth from 10% at the beginning of the process to 20% after 24 h (3 variant, Fig. 4) caused the decline in clinical fractions content.

Our research demonstrated that the production of controlled molecular weight dextran can be carried out by the combined use of dextransucrase and dextranase. The ratio dextransucrase/dextranase and sucrose concentration are important parameters in this process. The highest productivity of dextran synthesis (85%, including 50% of the clinical fractions) conducted in 10% sucrose solutions, and catalyzed by dextransucrase (0.4 U ml^{-1}) coupled with dextranase, was achieved at the concentration of the latter enzyme of 0.004 U ml^{-1}, and when this hydrolase was added 5h later than the transferase. To achieve total sucrose depletion when its concentration was increased to 15 or 20% (the initial concentration or attained via gradual supplementing) the longer reaction times were necessary (36 or 48h, respectively). The desired products pattern was observed when the dextranase was added to the reaction mixture 5h later than the dextransucrase. The clinical dextran was produced most efficiently when sucrose concentration was gradually increased in reaction mixtures. As much as 51.5% of clinical fractions were formed in 15% sucrose solutions, and approximately 46% – when this substrate concentration was 20%. A rise in sucrose concentration from 10 to 20% resulted in almost twice higher clinical dextran net amount. The only disadvantage in the latter case was the longer reaction time (48h), but the whole process required a relatively low energy input.

Day & Kim [7-9] used mixed culture of *Leuconostoc mesenteroides* NRRL B12b (dextransucrase producer) and *Lipomyces starkeyi* (dextranase producer) and dextran of the average molecular weight of 70 kDa was produced in high yield (70%). However, such a process was not ideal for the production of clinical dextran, due to the variety of different substances present in culture media and the difficulty in product purification.

Conclusions

Our experiments indicate that the clinical dextran can be efficiently produced by using both the compared methods, which employ either acceptor dextrans with definite molecular mass, or the dextranase. Because consumption of the latter enzyme is rather small, and it is easily available, thus the method with dextranase should be attractive for clinical dextran manufacturers.

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