concentration, % (w/v)); $_0$ is the intercept term; $_1$, $_2$ and $_3$ are the linear effects (main effect); $_{11}$, $_{22}$ and $_{33}$ are the quadratic effects; and $_{12}$, $_{13}$ and $_{23}$ are the interaction effects.

Table 1. *Experimental ranges of the independent variables in the experimental design*

Factors		0	1		
A: Distillery stillage concentration, % (v/v)	35	55	75	15	95
B: Sucrose concentration, % (w/v)	1.5	3	4.5	0	6
C: Mn^{2+} concentration, % (w/v)	0.006	0.012	0.018	0	0.024

The RSM was applied by using a statistical package, Design-Expert (Version 8, Stat-Ease, Inc., Minneapolis, US).

3. RESULTS AND DISCUSSION

3. 1. Evaluation of substrates for dextransucrase production

The chemical composition and metal ion contents in molasses and distillery stillage used for DS production in this study is presented in Tables 2 and 3, respectively.

Table 2. Chemical compositions of sugar beet molasses and distillery stillage

Parameter	Sugar beet molasses[a]	Distillery stillage[a]
Content of dry matter, wt.%	77.42 ± 0.89	11.55 ± 0.30
Content of total sugars, wt.%	54.80 ± 0.51	9.74 ± 0.04
Content of total nitrogen/ content of total protein, wt.%	1.48 ± 0.16	58.50 ± 0.12

[a]Values represent means ± standard deviation calculated from three determinations.

Table 3. Metal ions contents in sugar beet molasses and distillery stillage

Metals	Concentration ± SD, mg dm^{-3}		
	Sugar beet molasses[a]	Distillery stillage[a]	Optimal metal content for LAB [32]
Mg	340.00 ± 0.01	155.00 ± 0.01	480-972
Mn	9.52 ± 0.02	1.34 ± 0.01	
Ca	115000[b]	210.55 ± 0.03	
Fe	29.38 ± 0.04	3.02 ± 0.04	
Zn	9.06 ± 0.03	3.78 ± 0.01	
Na	135000[b]	398.02 ± 0.01	
Cu	0.80 ± 0.01	0.22 ± 0.05	

[a]All values represent means ± standard deviation calculated from three determinations, except
[b]which represents means calculated from three determinations without standard deviation.

Composition of molasses used in this study (Table 2) is in accordance with results reported in the literature [16]. Typically, molasses contains around 50 % of sucrose [17], 30 % non-sugar compounds and around 20 % water. In order to be used in the fermentation, molasses was diluted to the desired sugar content. Dilution of molasses has multiple advantages. Because of the high concentration of sugars, undiluted molasses acts inhibitory on bacterial growth. When molasses is diluted, the concentration of inhibitory components for bacterial growth, fermentation and/or production of DS also decreases. Additionally, dilution also decreases the concentration of salts, normally present in molasses but which can be harmful for microorganisms (Table 3). Among different studied concentrations of molasses (from 1 to 5 %) the best DS production was achieved at the concentration of 2.5 %, which corresponds to the sucrose concentration of 1 % in the fermentation media [29].

Analyses of molasses samples from different sugar factories over several seasons have shown that the total nitrogen content in molasses may vary considerably. According to the literature data, the total nitrogen content in molasses varies in the range of 0.8 to 2.2 %, calculated on the total mass of molasses [33]. This corresponds well with to results (Table 2). However, it has been determined that the betaine content is constant and in the range 33 to 43 % of total nitrogen [17,33]. Since microorganisms cannot use betaine in their metabolic pathways, the content of amino acids is a better criterion for assessing the suitability of molasses for fermentation. These amino acids are easily assimilated by

microorganisms. Diluted molasses was previously used for the growth of *L. mesenterioides*. The content of amino acids in 40-fold diluted molasses was sufficient for the growth of *L. mesenteroides* T3 but still very low and supplementation was needed for enhancement of the DS production [29]. Under these conditions, the contribution of molasses in the total nitrogen content in media is negligible and the origin of the molasses does not play a significant role.

On the other hand, a relatively high amount of proteins in distillery stillage (more than 50 % of dry matter, Table 2) suggests that it could be suitable as a substrate for growth of lactic acid bacteria (LAB). In a combined substrate based on distillery stillage and molasses, distillery stillage primarily acts as an additional source of α-amino nitrogen, which is of great importance since LAB are nutritionally demanding microorganisms, primarily in terms of organic nitrogen sources, such as free amino acids and peptides.

The presence and contents of metals in mixtures of distillery stillage and molasses after appropriate dilution are in correlation with the requirements of LAB [32] and are also below the inhibitory values. Chemical composition of mixtures of molasses and distillery stillage can provide necessary nutrients and fermentable sugars for the growth of *L. mesenteroides* T3 but for the enhancement of DS production it is necessary to add sucrose and Mn^{2+} [34]. In order to obtain the highest possible DS activity, the influence of different concentrations of sucrose and manganese together with the effects of different concentrations of distillery stillage were investigated by RSM.

3. 2. Fitting the process variables

A total of 20 randomized experiments, including six replicates as the center points were carried out according to the experimental design matrix (Table 4) derived from an optimal design for DS production.

Table 4. The design matrix and corresponding responses

Run	Independent variables			Response
	A / %	*B* / %	*C* / %	*Y* / U cm^{-3}
1	55	3.0	0.024	2.322
2	35	4.5	0.018	2.544
3	35	1.5	0.006	1.743
4	55	3.0	0.000	1.862
5	55	3.0	0.012	2.172
6	55	3.0	0.012	2.179
7	95	3.0	0.012	1.485
8	55	3.0	0.012	2.124
9	55	3.0	0.012	2.201
10	75	4.5	0.006	2.054
11	75	1.5	0.018	1.551
12	35	4.5	0.006	2.176
13	55	0.0	0.012	1.062
14	75	1.5	0.006	1.192
15	35	1.5	0.018	1.506
16	15	3.0	0.012	1.597
17	55	3.0	0.012	2.001
18	55	3.0	0.012	1.98
19	75	4.5	0.018	3.077
20	55	6.0	0.012	2.664

A - stillage concentration; *B* - sucrose concentration; *C* - manganese concentration; *Y* - DS activity

For the three examined factors, the CCD model efficiently designed a second order response fit for the surface. The quadratic model was found to be the most suitable model. The statistical significance of the regression model was evaluated by the analysis of variance (ANOVA) (Table 5). For regression analysis, the model was modified by removing the effect of non-significant factors by using backward reduction and the quadratic equation that predicts the maximum yield of DS production:

$$Y = 2.13 - 0.02A + 0.44B + 0.15C + 0.11AB + 0.16AC + 0.16BC - 0.14A^2 - 0.055B^2 \quad (2)$$

where *Y* (DS activity, U/ml) is the response and *A* (stillage concentration, %), *B* (sucrose concentration, %) and *C* (Mn^{2+} concentration, %) were independent variables while *AB*, *AC* and *BC* present interactions between variables *A*, *B* and *C*.

Table 5. The analysis of variance (ANOVA) for the quadratic model presented by Eq. (2)

	F-value	p-value Prob > F
Model	50.26	< 0.0001[a]
A	0.57	0.4673[b]
B	278.22	< 0.0001[a]
C	32.98	0.0001[a]
AB	9.37	0.0108[a]
AC	17.44	0.0015[a]
BC	17.94	0.0014[a]
A^2	43.14	< 0.0001[a]
B^2	7.18	0.0214[a]
Lack of fit	1.41	0.3619[b]
R-Squared	0.9734	
Adjusted *R*-squared	0.9540	
Predicted *R*-squared	0.8755	
C.V.%	5.36	
Adequate precision	26.981	

[a]Significant coefficient ($P < 0.05$); [b] Non-significant coefficient

As it can be seen in Table 5, the significant factors that influence the response and have a p-value (Prob > F) < 0.05 were *B* and *C*, the quadratic coefficients of *A* and *B*, as well as the interactions *AB*, *AC* and *BC*. Adequacy of the model for predicting the DS production can be indicated by the non-significant F-value for the lack of fit (1.41) compared to the pure error. The following determination coefficients: R-squared, adjusted R-squared and predicted R-squared were calculated to check the fit of the model. The obtained values of R-squared coefficients were close to 1 which showed a good correlation between the predicted and observed values (Fig. 1A). The actual values were measured response data for a particular run, and the predicted values were evaluated from the model. The adequate precision value of 26.981 was greater than 4, which indicates that the signal was adequate. The value of the coefficient of variation (C.V.) of 5.36 indicated a high degree of precision and reliability of the experimental values, suggesting that the model was reliable and reproducible [35].

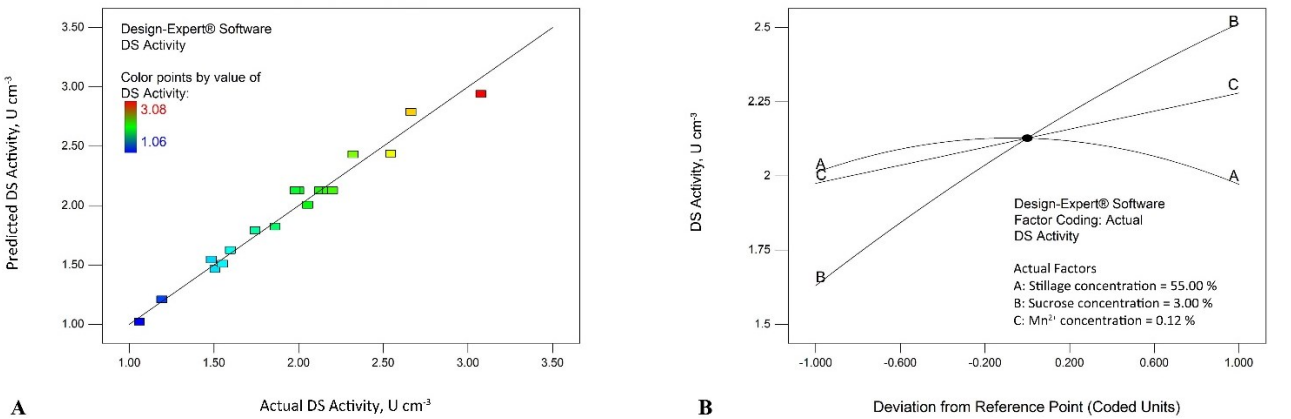

Fig. 1. Plots of: (A) the relationship between the experimental and predicted values for the DS production and (B) the perturbation of all the variables

3. 3. Influence of process variables on DS production

The presence of carbon and nitrogen sources is necessary for the bacterial growth and the synthesis of enzymes. In preliminary investigations, medium with molasses and stillage was compared with medium supplemented only with molasses, proving that the combined medium was a better substrate for bacterial growth reaching higher DS activity, and thus, statistical optimization of this medium was performed.

The influence of three process variables on the DS production of *L. mesenteroides* T3 was examined. Our preliminary investigations [34] on the influence of temperature on the enzyme activity showed that the optimal temperature was 23 °C for the maximal enzyme production, and thus this temperature was fixed in further experiments.

The DS activity in fermentation medium obtained under the tested conditions was in the range from 1.062 to 3.077 U cm^{-3}. According to the analysis of the experimental data and derived regression model, it can be concluded that two of three linear regression coefficients (β_2 and β_3) are significant (Table 5 and Eq. (2)). On the other hand, two quadratic regression coefficients (β_{11} and β_{22}), are significant and negative, and therefore, the influence of corresponding parameters: stillage and sucrose concentration on the DS production can be described as a quadratic function with a maximum value. Moreover, all interactions between the examined parameters proved to be significant and positive. The significance of each coefficient was determined by p-values which are listed in Table 5. The influence of different variables on the DS production was in the following order: sucrose concentration (*B*) > Mn^{2+} concentration (*C*) > stillage concentration (*A*) (Table 5). Interactions between the stillage concentration and Mn^{2+} concentration, *AC*, and the sucrose concentration and Mn^{2+} concentration, *BC*, were of higher significance than the interactions between the stillage concentration and sucrose concentration *AB*.

The main advantage of the response surface methodology is the possibility to evaluate interactions between tested variables and define the optimum values of the variables such that the response is maximized. The sucrose concentration is the most significant factor which positively affected the DS production. With the increase in sucrose concentration up to 6 %, the DS production increased. In conducted experiments, it was observed that the addition of sucrose at a concentration of 3.0 % to the production medium led to an increase in the DS production by approximately 50 %, when stillage and Mn^{2+} concentrations were maintained constant. The highest enzyme activity of 3.077 U cm^{-3} (according to the CCD model) was achieved in the medium with the addition of 4.5 % of sucrose. But according to the optimal conditions for the DS production, the maximum enzyme activity was achieved when sucrose is added at the concentration of 5.30 % to provide the total sucrose concentration of 7 % in the fermentation medium. Similar sucrose concentration appears to be optimal for DS synthesis by other dextran-producing strains such as *L. mesenteroides* NRRL-B640, as seen in other studies [36]. In order to visualize influence of the independent variables (*A*, *B* and *C*) on the DS production, Eq. (2) was expressed as a response surface plot (Fig. 2). The interaction between the sucrose and stillage concentrations is presented in Fig. 2A. The maximal DS concentration was achieved at the highest concentration of sucrose (6%) and in the range of higher stillage concentrations (55-75 %). Also, it could be noticed that there was the increase in DS production when higher concentrations of sucrose (5-6 %) and Mn^{2+} (0.018-0.024 %) were used (Fig 2C).

According to the Eq. (2) the Mn^{2+} concentration (*C*), as a single factor, has a positive influence on the DS production and exhibits significant positive interactions: Mn^{2+} - stillage (*AC*) and Mn^{2+} - sucrose (*BC*) (Fig. 2 B, C). By comparing the enzymatic activity in the medium with the highest DS activity (Run 19, Table 4), with the medium that contained the same concentrations of stillage and sucrose (Run 10, Table 4) we concluded that the increase in the concentration of Mn^{2+} ions increased the DS activity for 33 %.

The importance of different ions with regard to the enzyme production processes is generally accepted. Purama and Goyal [36] observed a 12 % increase in the enzyme production with the increase in the concentration of $MnSO_4$ from 0.001 (control) to 0.005 % for *L. mesenteroides* NRRL B-640. The essential role of Mn^{2+} ions for the DS production by *L. mesenteroides* strains was also reported [1]. The addition of amino acids, Mg^{2+} and Mn^{2+} ions stimulated the growth of most *Leuconostoc* strains [23]. It has been also shown that Mn^{2+} suppressed the inhibitory effect of aeration on the growth of *L. mesenteroides* UD-23 [23]. High requirements of Mn^{2+} ions could be explained by its interaction with enzymes and the ability to scavenge toxic oxygen radicals resulting in a protective role. In our previous studies on the DS production, Mn^{2+} ions also showed a positive effect on the activity of partially purified DS obtained from *L. mesenteroides* T3 [34].

In the present experiments, stillage was used as a source of nitrogen. From the perturbation plot (Fig. 1B), the influence of individual factors on the DS production can be seen (*Y*). A sharp curvature, a function with a maximum value, for stillage concentration (*A*) (Fig 1.B) shows that the DS production yield is highly sensitive to this parameter and correspondingly the quadratic regression coefficient has a negative value (Eq. 2). With increasing the concentration of stillage, the DS

production yield is increasing, until a maximum is reached after which a further increase in stillage concentration leads to the decrease in DS production. In conducted experiments, the medium with the highest DS activity (Run 19, Table 4) contained approximately 2.5 % of nitrogen, which is similar to the commercial De Man, Rogosa and Sharpe (MRS) medium. Residual yeast from bioethanol production in distillery stillage contributes as a source of assimilative nitrogen and thermo stable vitamins, affecting the efficiency of sugar utilization and promoting the growth of LAB [37].

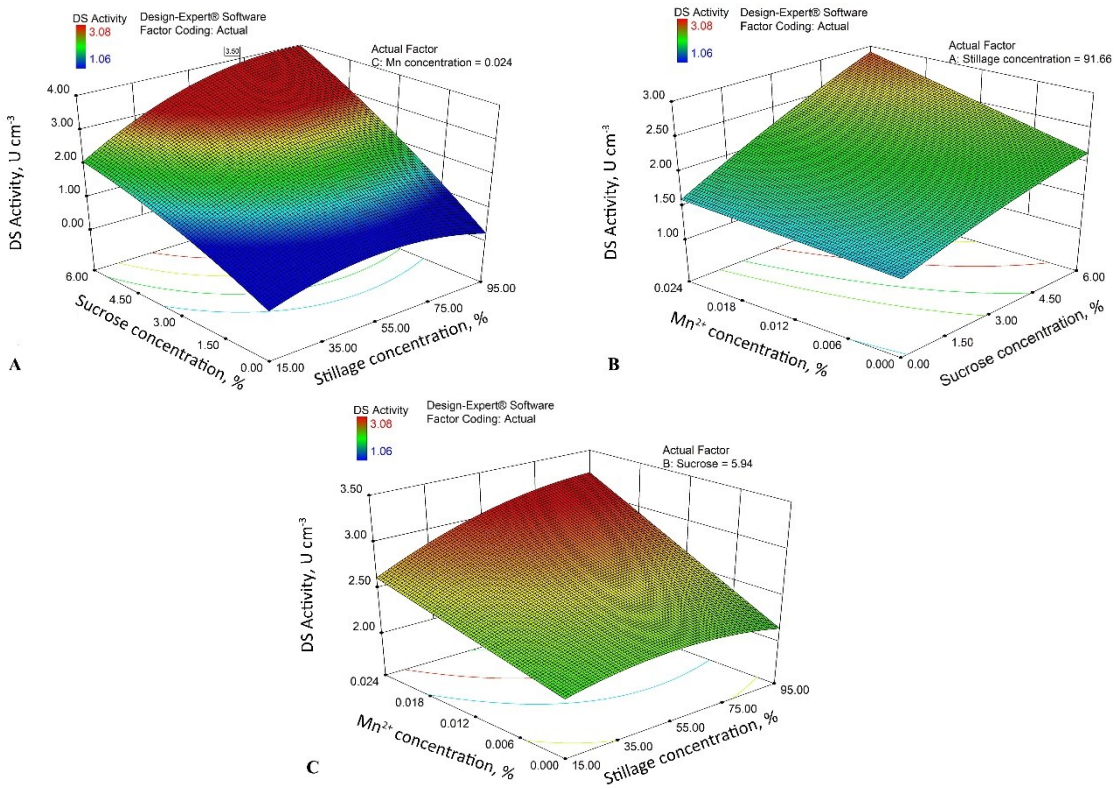

Fig. 2. Surface plots of interactive effects of: (A) stillage concentration and sucrose concentration (AB), (B) sucrose concentration and Mn^{2+} concentration (BC) and (C) stillage concentration and Mn^{2+} concentration (AC)

There are other studies with waste materials as substrates for DS and dextran productions [10, 40, 41]. According to literature data, molasses was used several times for production of DS and other enzymes. For example, *L. mesenteroides* FT 045B produced DS with the maximum activity (4.03 U cm^{-3}) after 24 h of fermentation while growing on molasses with the addition of corn steep liquor as the nitrogen source [40]. On the other hand, low DS activity 4.3 DSU cm^{-3} h^{-1} (where one DSU was defined as the enzyme quantity that converts 1.0 milligram of sucrose into fructose and dextran in 1.0 h) obtained from *Lactobacillus acidophilus* was reported on molasses as the sole carbon source [41]. In our previous work, we optimized conditions for enhancement of DS production (2.02 U cm^{-3}) on molasses using sugar beet pulp as a support for immobilization of *L. mesenteroides* T3 [29].

In the present study, a 60 % higher DS production (3.391±0.131 U cm^{-3}) in comparison to our previous study has been obtained on cheaper and abundant substrate.

3. 4. Validation of the model

The objective of this study was to find the optimal medium composition, using two waste materials, for DS production by *L. mesenteroides* T3. In order to validate the obtained model one point was selected from the numerical optimization results. The experiment was conducted with 64.33 wt % stillage, 5.30 wt % sucrose and 0.022 wt % Mn^{2+}. The predicted value for the outcome DS activity was 3.498 U cm^{-3} with 95 % prediction interval (PI) 3.098 – 3.898. The

measured value for the parameter fitted within the 95 % PIs, and was very close to the most probable predicated value for the DS activity (3.391±0.131 U cm^{-3}), showing that the model is reliable.

The nitrogen: carbon ratio has an important role in optimization of the medium composition for the DS production. After calculation of the total nitrogen and carbon contents in the medium that provided the highest DS activity, the obtained nitrogen: carbon ratio was approximately 0.67:1 (0.85% molasses + 5.30% sucrose + 0.8% stillage or a total of 7% for carbon and 4.7% for nitrogen concentrations). According to literature [4] this is the most suitable nitrogen: carbon ratio for DS production.

4. CONCLUSION

Current trends in the enzyme production include the use of low-cost or waste substrates. Revalorization of agro-industry waste as a substrate for biotechnological production fits within the sustainable development goals [42]. We have demonstrated that two waste substrates, distillery stillage and sugar beet molasses could be combined as cheap and renewable sources of nitrogen, vitamins, minerals and fermentable sugars for the growth of *L. mesenteroides* T3 and for the DS production. The applied optimization process by CCD has shown that 60 % increase in the DS activity (3.391±0.131 U cm^{-3}) has been obtained on a cheaper and abundant substrate, as compared to our previous study. Manganese and sucrose are identified as key linear correlating components in media optimization for the DS production. Development of a process for DS production on waste materials with possible reductions of expenses can have both an economic and an ecological significance.

This study proves potentials for using wastes from one industry as the substrates for obtaining valuable biotechnological products in the other industry in accordance with principles of circular economy. It could serve as a basis for the development of a process for DS production with possible reduction of expenses and environmental footprint.

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SAŽETAK

Iskorišćenje nus-proizvoda agro-industrije za proizvodnju dekstransaharaze pomoću bakterije *Leuconostoc mesenteroides* T3: optimizacija procesa metodom odzivnih površina

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(Naučni rad)

Dekstransaharaza (DS) je glukoziltransferaza (E. C. 2.4.1.5.) koja katalizuje prenos ostataka glukoze iz saharoze u polimer dekstrana, pri čemu se oslobađa fruktoza. Ovaj enzim je povezan sa širokim spektrom primene dekstrana i oligosaharida. Proizvodnja dekstransaharaze pomoću bakterije *Leuconostoc mesenteroides* T3 optimizovana je metodom odzivnih površina korišćenjem centralnog kompozitnog dizajna. Za optimizaciju su izabrane tri promenljive: koncentracija džibre, koncentracija saharoze i koncentracija jona mangana. Rezultati su pokazali da koncentracije saharoze i jona mangana imaju pozitivan linearni efekat na proizvodnju DS dok su sve interakcije (džibra-Mn^{2+}, džibra-saharoza i saharoza-Mn^{2+}) imale značajan uticaj na proizvodnju DS. Na osnovu eksperimentalnih podataka i numeričke optimizacije, dobijen je maksimalni prinos DS od 3.391 ± 0.131 U cm^{-3} u podlozi sa 64.33 % džibre, 5.30 % saharoze i 0.022 % jona mangana. Naše istraživanje otkrilo je da se džibra u kombinaciji sa melasom šećerne repe kao i saharozom i dodatkom jona mangana može koristiti kao dragocena hranjiva komponenta za rast bakterija mlečne kiseline i proizvodnju DS. Takođe, uzimajući u obzir poreklo supstrata, upotreba industrijskih nusproizvoda na ovaj način ima veliku ekološku važnost.

Ključne reči: bakterije mlečne kiseline; dekstran; proizvodnja enzima; destilerijska džibra; melasa