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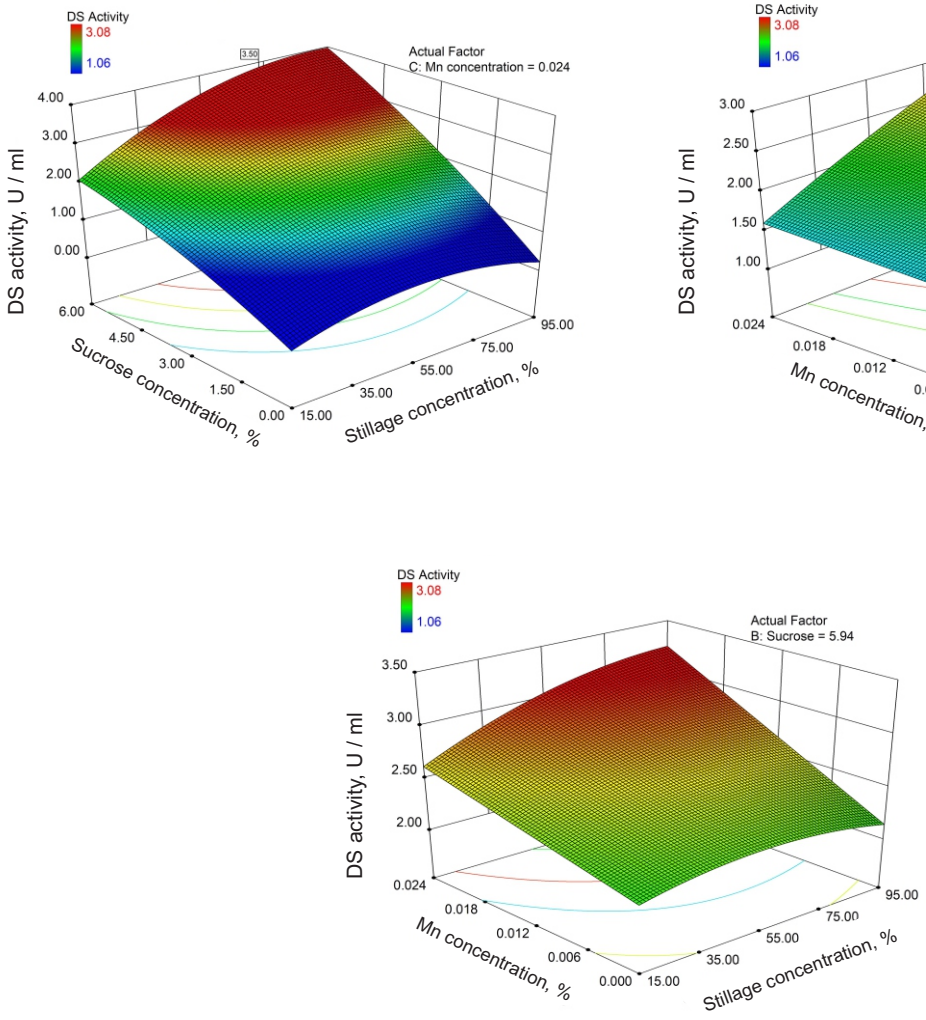

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SADRŽAJ/CONTENTS

Utilization of agro-industrial by-products as substrates for dextransucrase production by *Leuconostoc mesenteroides* T3: process optimization using response surface methodology

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Abstract

Dextransucrase (DS) is a glucosyltransferase (E. C. 2.4.1.5) that catalyzes the transfer of glucosyl residues from sucrose to dextran polymer and liberates fructose. This enzyme isassociated with a wide application range of dextran and oligosaccharides. DS production by Leuconostoc mesenteroidesT3 was optimized using a Central Composite Design under the Response Surface Methodology. Three variables were chosen for optimization: distillery stillage, sucrose and manganese concentration. The results showed that sucrose and manganese concentrations had a positive linear effect on DS production while all variable interactions (stillage-manganese, stillage-sucrose, and sucrose-manganese) had significant influences on the DS production. The maximal DS yield of 3.391±0.131 U cm^{-3}, was obtained in the medium with 64.33 % distillery stillage concentration, 5.30% sucrose concentration and 0.022 % manganese concentration. Our study revealed the potential of distillery stillage combined with sugar beet molasses, supplemented with sucrose and manganese to be employed as a valuable medium growth for lactic acid bacteria and production of DS. Also, taking into consideration the origin of the substrates, utilization of industrial by-products in this way has a great environmental relevance and is in accordance with circular economy.

Keywords: headspace; gas chromatography; alcoholic beverages; cosmetics.

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1. INTRODUCTION

Dextransucrase (DS) is a glucosyltransferase (E. C. 2.4.1.5) that catalyzes the transfer of glucosyl residues from sucrose to dextran polymer and liberates fructose [1]. This enzyme also catalyzes the so-called acceptor reaction, where in the presence of suitable low molecular weight molecules, for example maltose, the transfer of glucosyl units is redirected from dextran to oligosaccharide synthesis [2]. When water molecule itself is an acceptor, only the reaction of sucrose hydrolysis to glucose and fructose takes place. DS is an extracellular enzyme produced by several species belonging to the genera *Lactobacillus*, *Leuconostoc* and *Streptococcus* [3]. It has been shown that the DS expression is constitutive in *Streptococcus* strains, whereas in *Leuconostoc* spp. DSs can only be produced upon sucrose induction [4,5]. DS is commercially employed in dextran production. Dextrans and their derivatives have been profusely used in food, clinical, pharmaceutical, fine chemicals, cosmetics and agricultural industries [6, 7]. Worldwide market for dextran is expected to reach 220 million US$ in 2024 with the annual rate of 4,2 % [8]. To date, different combinations of cultivation parameters for DS producing strains have been used to obtain the maximal activity of DS.

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Various industrial by-products and wastes have a great potential for utilization as raw materials for production of biomolecules, especially enzymes. Taking into consideration that production of enzymes is expensive, mainly due to the high price of the medium and the substrates for microbial fermentations, current challenges in the production of enzymes include testing the possibilities to use wastes or low-cost materials. Several by-products, including sugar beet molasses [9], carob pod extract and cheese whey [10], and cashew apple juice [11] have already been studied as low-cost substrates for the DS production.

Distillery stillage, also termed distillery wastewater, is the major by-product from the distillation of ethanol following fermentation of carbohydrates. The production of bioethanol from biomass, whether from sugar crops, starch crops, dairy products or cellulosic materials, at the same time results in the production of stillage, which exhibits a considerable pollution problem [12]. An average amount of stillage produced in the bioethanol process is approximately 13 L per L of bioethanol [13]. Taking into account a high BOD_5 values and low pH values, its storage represents an important ecological problem in the industrial facilities and the high costs of treatment prior to disposal into watercourses seriously affect the viability and profitability of the process [14]. However, due to its complexity and origin, the stillage from bioethanol production on starch substrates (corn, rye, wheat, potato, etc.) could be a valuable source of nitrogen, vitamins and minerals, which are necessary for the growth of microorganisms [13].

Sugar beet molasses is a by-product of the sugar industry. It is especially attractive, not only because of its low price but also because of the presence of a number of components including minerals, organic compounds and vitamins, which are very useful for the fermentation process [15]. Molasses contains up to 54 % of sugars [16] among which sucrose is the most abundant followed by small quantities of glucose, fructose and raffinose [17]. It is also a valuable source of growth substances such as pantothenic acid, inositol, trace elements, and, to a lesser extent, biotin [18]. Serbia has significant surpluses of sugar beet and in 2011 the export of sugar beet molasses was 59.655 t [19]. Based on its properties, molasses can be considered as a promising starting material or substrate for microbial fermentation [20].

Certain metallic ions like Mg^{2+}, Ca^{2+}, and especially Mn^{2+} are essential for DS production and bacterial growth according to literature [21,22]. *Leuconostoc* spp. are known to be micro-aerophilic microorganisms [1] and $MnSO_4$ decreases the oxygen toxicity in the *L. mesenteroides* [23].

The Response Surface Methodology (RSM) represents a useful tool for studying the effects of several factors on one or more responses by varying them simultaneously. In addition, the optimal response is calculated based on the experimental data obtained from a limited number of experiments [24]. RSM has been applied in many areas of biotechnology such as optimization of culture medium, enzyme synthesis, aqueous two-phase separation of proteins, glucan production, *etc.* [25]. Considering possible variations in composition of different agro-industrial wastes, it is necessary to do composition screening (carbon and nitrogen determination) every time when a new batch is obtained. It is also very important to know which raw material is the basis of distillery stillage. For example, bread stillage has significantly more nitrogen than potato stillage [26]. RSM is therefore a useful tool for optimization of a media based on agro-industrial wastes for bacterial growth.

The objective of this study was to test two cheap and abundant by-products from bioethanol production and sugar industry for DS production by *L. mesenteroides* T3. The molasses and distillery stillage as cheap agro-industrial by-products were supplemented with sucrose and manganese to enhance the DS production. The Central Composite Design (CCD) under RSM was used for optimization of fermentation parameters: concentrations of stillage, sucrose and manganese to obtain a maximal DS activity.

2. EXPERIMENTAL

2. 1. Microorganism for dextransucrase production

The microorganism used in this study is *Leuconostoc mesenteroides* T3 natural isolate from water kefir grain, identified as *L. mesenteroides* as described earlier [27].

2. 2. Inoculum and medium preparation for dextransucrase production

For inoculum preparation the organism was grown in slightly modified medium reported by Tsuchiya et al. [28] and it contained: sucrose (Lach-Ner, Czech Republic), 40.0 g dm^{-3}; yeast extract (Torlak, Serbia), 20.0 g dm^{-3}; K_2HPO_4 (Centrohem, Serbia), 20.0 g dm^{-3}; $MgSO_4 \cdot 7H_2O$ (Kemika, Croatia), 0.2 g dm^{-3}; $MnSO_4 \cdot H_2O$ (Kemika, Croatia), 0.01 g dm^{-3}; NaCl (Zorka, Serbia), 0.01 g dm^{-3}; $CaCl_2$ (Lach-Ner, Czech Republic), 0.02 g dm^{-3}; $FeSO_4 \cdot 7H_2O$ (Zorka, Serbia), 0.01g dm^{-3}. All chemicals were of analytical grade. The medium pH was adjusted to 6.9 by using orthophosphoric acid. Erlenmeyer flask cultures were grown for 16h at 23°C under static conditions.

Sugar beet molasses and distillery stillage were obtained from Swan lake d.o.o, Kovin, Serbia and Reahem d.o.o., Srbobran, Serbia, respectively. Fermentation medium was prepared with the fixed concentration of molasses (2.5 wt.%) that was previously defined as optimal for DS production by the same strain [29]. Stillage was added to the molasses medium at different concentrations according to the values given by RSM.

2. 3. Fermentations

All experiments were performed in 100 cm^3 Erlenmeyer flasks with a medium volume of 20 cm^3. Molasses in 2.5 wt.% concentration was used as a liquid medium for fermentations. In each flask with molasses, stillage was added, separately, in a defined concentration range (15-95 % v/v). Sucrose as a main substrate for DS production was also implemented into the medium in the range from 0-6 % (w/v). Manganese ion was varied in the range from 0-0.024 % (w/v). K_2HPO_4 was added as a buffer substance in a concentration of 20 mM. After sterilization at 121 °C for 20 min, an overnight bacterial culture was inoculated into fresh medium at 23 °C for 12 h under shaking (180 rpm). The culture medium was centrifuged at 6000*g* for 15 min to remove the cells. The crude cell-free supernatant was analyzed for DS activity.

2. 4. Analytical methods

The dry matter content was determined by a standard drying method in an oven at 105°C to constant mass [24]. The total nitrogen content in molasses was estimated by the Kjeldahl method [24]. The protein content for stillage was estimated by the Kjeldahl method as the total nitrogen and multiplied by the factor of 6.25 [30]. In order to determine the concentration of reducing sugars, the sample solution was hydrolyzed by HCl at 100°C for 10 min and neutralized with NaOH solution. Then, the concentration of reducing sugars was estimated by the 3,5-dinitrosalicylic acid method (DNS) [31]. The metal contents in the liquid distillery stillage and molasses were determined by atomic absorption spectroscopy (Perkin Elmer Analyst 200, Waltham, USA).

The DS activity assay was carried out in a 450 mm^3 reaction mixture containing 10 % (w/v) sucrose, 20 mM sodium acetate buffer (pH 5.4) and 50 mm^3 cell free supernatant at 30 °C for 15 min. The enzyme activity was determined by measuring the concentration of released reducing sugars by the DNS method, using fructose as a standard [31]. The absorbance was measured at 540 nm by using a spectrophotometer (Ultrospec 3300 pro, Amersham Biosciences). One unit of DS activity was defined as the amount of enzyme releasing 1 µmol of reducing sugars per minute.

2. 5. Experimental design

Based on preliminary "one variable at the time" experiments (data not shown) a CCD was chosen to examine the effects of three independent variables: distillery stillage concentration (A), sucrose concentration (B) and manganese ion concentration (C) within the defined ranges that favored optimal feedback of the DS production response. Each factor in this design was coded in five different levels (Table 1). The data obtained from CCD were analyzed by multiple regressions to fit to a second-order polynomial regression model containing the coefficients of linear, quadratic, and two factor interaction effects.

The model equation of response (Y) on three independent variables (X1, X2 and X3) is given in the following equation:

$$Y = \beta_0 + \beta_1 X1 + \beta_2 X2 + \beta_3 X3 + \beta_{12} X1X2 + \beta_{13} X1X3 + \beta_{23} X2X3 + \beta_{11} X1^2 + \beta_{22} X2^2 + \beta_{33} X3^2 \quad (1)$$

where Y (DS activity, U cm^{-3}) is the dependent variable or predicted response associated with each factor level combination; X1 (distillery stillage concentration, % (v/v)), X2 (sucrose concentration, % (w/v)), and X3 (manganese ion

concentration, % (w/v)); β_0 is the intercept term; β_1, β_2 and β_3 are the linear effects (main effect); β_{11}, β_{22} and β_{33} are the quadratic effects; and β_{12}, β_{13} and β_{23} are the interaction effects.

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

Factors	−1	0	1	Axial (−α)	Axial (+α)
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	≤110
Ca	115000[b]	210.55 ± 0.03	≤8000
Fe	29.38 ± 0.04	3.02 ± 0.04	≤4
Zn	9.06 ± 0.03	3.78 ± 0.01	<20
Na	135000[b]	398.02 ± 0.01	≤4000
Cu	0.80 ± 0.01	0.22 ± 0.05	<19

[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].

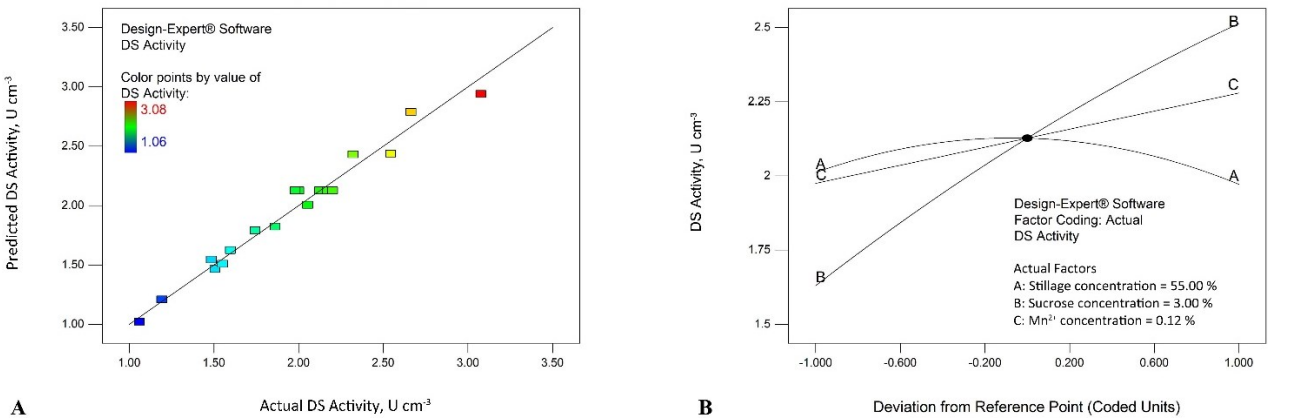

Fig. 1. Plots of: (A) the relationship between the experimental and predicted values for the DS production and (B) the perturbation of all the variables

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].

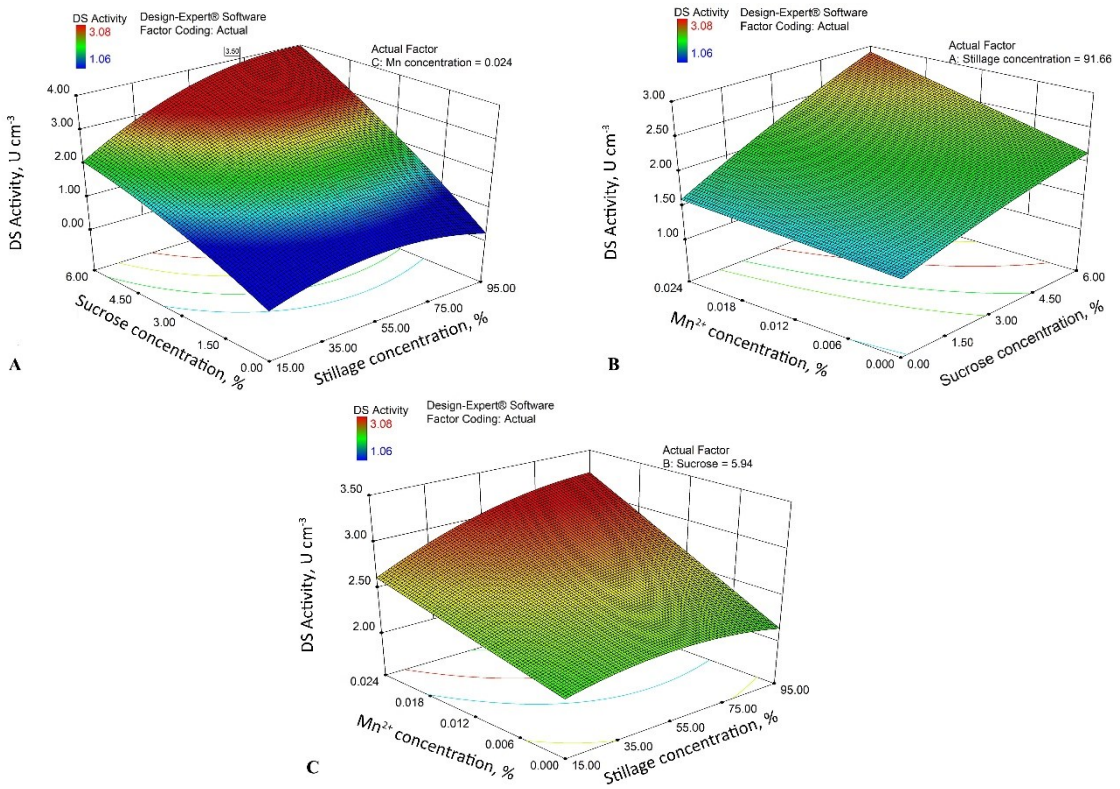

Fig. 2. Surface plots of interactive effects of: (A) stillage concentration and sucrose concentration (AB), (B) sucrose concentration and Mn^{2+} concentration (BC) and (C) stillage concentration and Mn^{2+} concentration (AC)

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

Miscanthus x giganteus as a building material - lightweight concrete

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Abstract

A perennial plant *Miscanthus* x *giganteus* has found its habitat and multiple applications in Europe, despite the fact that it originates from Asia. This study presents the potential use of this plant in new lightweight concrete materials so-called bio-concretes. The above-ground part of the plant was harvested, dried, crushed, and mixed with binders in different proportions. After casting and drying, the samples were characterized physical and mechanical properties. The results have shown that the sample with a higher content of binders while smaller miscanthus granulation and casted in molds under higher pressure exhibited the highest values of the compressive strength and density. In specific, the density was in the order of magnitude of that reported for other types of lightweight concrete with organic fillers, such as sawdust-based concrete ("Durisol"), which further justifies the use of miscanthus for these purposes.

Keywords: bio-concrete, biomass, lignocellulosic fibers, yield, green-building, agro-energy crops.

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TECHNICAL PAPER

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1. INTRODUCTION

Miscanthus x *giganteus,* originally from Asia, has been grown in Europe since the 1930s. It is planted in spring when the soil temperature rises above 10 °C, it grows during the summer, and it is harvested in the late autumn. Harvesting is recommended after the third vegetative season due to low yields in the first and second year after planting [1]. The lifetime of this plant is estimated to last up to 20 years [2]. After the third year, miscanthus reaches the height of about 3 m, so that the aboveground biomass is collected, dried, and further treated depending on the purpose. This type of miscanthus shows the properties of a good soil remediator, so its application is widespread to phytoremediate contaminated sites [3]. Miscanthus plantations create additional habitats for plant and animal species, while the biomass can be used in the production of pellets and briquettes. In addition, as we have observed in the demonstration field FPE Futura in the village Noćaj near Sremska Mitrovica (Fig. 1), the upper parts of miscanthus are eaten by wild horses, goats, and cows, indicating that this plant could be used as animal feed as well.

Miscanthus shows higher productivity in humid environments, and therefore higher yields can be expected in wetlands [4], while at a lack of water the annual yield decreases [5].

Construction is currently a sector with high impacts on the environment and its sustainability, which is reflected primarily through the exploitation of non-renewable natural resources, energy consumption, land occupation and the like [6]. Cement concrete is the most widely used engineering material due to its excellent resistance to water, simple formation of structural concrete elements in a large variety of shapes and sizes, and, usually, its immediate availability [7]. Concrete consists of a binding medium, which is usually cement, water, aggregates, and reinforcing steel bars, which are expensive and for whose individual production a large amount of energy is consumed [8].

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Figure 1. Miscanthus x giganteus at the Futura faculty field

Various literature references mention the use of lignocellulosic plant species in construction materials as constructional, non-constructional or thermal insulation materials, which indicates possibilities for the use of miscanthus for similar purposes [7-14]. Another reason is the growing demand for environmentally friendly building materials imposing. Reinforcement of building materials with natural fibers was reported in the 1990s [29]. It was shown that fibers originating from agriculture, with a lignocellulosic composition like miscanthus, can be used alone or to strengthen materials of different origins (earth materials and cement composites) [9]. The advantages of natural fibers are their wide distribution in the environment, satisfactory physical and mechanical performances [10], as well as possibilities for production from renewable sources or waste biomass. Based on the specific properties of these materials, primarily low density, and compressive strength, they can be compared to materials based on artificial fibers [11].

It was reported that miscanthus was incorporated into building materials as insulation, composite materials, fiberboard, miscanthus – concrete, and miscanthus biocomposites [12]. Examination of acoustic absorption properties and interactions between this plant material and Portland cement, demonstrated that the sound absorption of the investigated miscanthus lightweight concrete was dramatically improved with the increasing miscanthus content due to the existence of closed internal pores in the composites [13].

It was shown that mixtures of miscanthus aggregates are comparable to other lightweight concrete mixtures [14]. Accordingly, the aim of the present work was to obtain such mixtures, determine physical and mechanical properties of the obtained concrete samples and compare the obtained results to other types of lightweight concrete with organic filler.

2. MATERIALS AND METHODOLOGIES

2. 1. Materials

Miscanthus was harvested after the third year from planting at the demonstration field of the Faculty for Applied Ecology Futura in the village Noćaj near Sremska Mitrovica, Serbia, by special harvesters, modified as compared to those used for corn due to the miscanthus structure [15]. Thus, the collected biomass from the field can be directly shredded or baled and stored. Safe storage of miscanthus is possible after drying the biomass up to 15 % of moisture [16], which can be achieved in the field or in a ventilated warehouse [17]. In this research, a certain amount of miscanthus used for testing was air-dried for one month in the summer period in a ventilated room, and then crushed in a laboratory chopper, into fractions of dimensions 5 to 10 mm. This procedure eliminated the significant presence of dust, which can interfere with binding performances of the fibers.

Hydrated lime ($Ca(OH)_2$) and metakaolin (Metaver™ N, Newchem AG, Swithzerland) were used as a binder to obtain hydraulic lime. Hydrated lime is a multifunctional additive that improves the durability of concrete and asphalt and it was obtained by mixing calcium oxide (CARMEUSE SRBIJA" doo, Jelen Do Serbia, granulation 0.1 – 1.5 mm) with water.

Metakaolin "Metaver™ N" is easily mixed in producing soft plastic consistence, easy to work with. The specific density is 2,600 kg m^{-3} and particle size distribution d_{50} ~3.4 – 4.5 μm, d_{95} ~12 - 18 μm. In relation to its reactivity, it can be qualified as „rapid", because together with lime and water the setting will occur in about 4 h (as stated by the producer). Calcium sulfate dehydrate gypsum ($CaSO_4 \cdot 2H_2O$, Volari, Bosnia and Herzegovina) with the granulation of 0.5 – 1.5 mm was used as a plasticizer. Cement with special characteristics (CEM II 52.5, Duracrete® basic, SCHWENK Zement KG, Germany) was selected for this type of research.

2. 2. Preparation of mixtures

Four composite material mixtures (marked I – IV) with different component mass fractions were prepared (Table 1). It should be noted that in the mixture IV, miscanthus chips of the approximate size of 5 mm were specifically extracted and used for preparation.

Table 1. Composition of prepared mixture series

Series	*m* / g (wt.%)					
	Miscanthus	Hydated lime	Portland cement	Metkaolin	Gypsum	Water
I	1000 (25)	700 (17.5)	/	700 (17.5)	100 (2.5)	1500 (36.6)
II	3000 (70.1)	150 (3.5)	300 (7.1)	/	30 (0.7)	750 (17.7)
III	2000 (71)	400 (14.3)	/	200 (7.14)	200 (7.14)	/
IV*	1000 (25)	700 (17.5)	/	700 (17.5)	100 (2.5)	1500 (36.6)

*Miscanthus chips of 5 mm in size were used and additional compression of the material in the mold was performed

The fractions of miscanthus chips were 25 and 71 wt.% while the binder fractions were in the ranges 3.5 – 17.5 wt.% for the hydrated lime,7.14 – 17.5 wt.% for metakaolin, 7.1 wt.% for Portland cement (in just one series) and 0.7 – 7.14 wt.% for gypsum.

Basic lime binders were used for the composite preparation since previous research has shown that *Miscanthus* x *giganteus* is resistant to the basic environment and the presence of silicates [18]. The measured components were placed in a plastic bucket and manually mixed, followed by mixing by a hand construction mixer. Mixing in a large construction mixer failed to adequately unite the material, due to the miscanthus fibrous structure. Water was added according to the need to obtain uniform mixtures.

The obtained mixtures were poured into cube-shaped steel molds (15×15×15 cm) with a working volume of 1 dm^3 and manually compacted by a wooden stick, with the samples in the last series compacted as hard as possible by this method. Compaction of the samples under a press was not successful since it produced too dry samples. The samples were dried in molds for 7 days followed by drying in air for another seven days. Three samples were produced in each experimental series.

2. 3. Physical and mechanical characterization

The dried samples were characterized regarding the dimensions and density followed by determination of the compressive strength after 14 days (Fp_{14}) and water absorption.

Density of the samples was determined by weighing the samples after a certain period (in this case 14 days) and measuring dimensions with a precision of 0.5 mm.

Compressive strengths were determined using a fully automatic machine *Tonipact 3000* (Toni Technik, Germany) for standard-compliant compressive strength tests (standard EN 12390-3), with the capacity of 3000 kN. The device is located in a material testing laboratory, at a constant temperature (T = 24 °C) and humidity (64 %). There was no need to change the ambient conditions during the examination. The samples (10 cm cubes) were measured in duplicates.

Water absorption of samples was tested by the gradual immersion method according to the standard procedure SRPS B.D8.011. The test consists of the following stages (Fig. 2):

- dipping the specimen to 1/4 height and keeping it in water for 1 h,
- immersion of the sample to 1/2 height and holding in water for 1 h,
- immersion of the sample to 3/4 height and holding in water for 20 h,
- full immersion of the sample and keeping in water for 2 h.

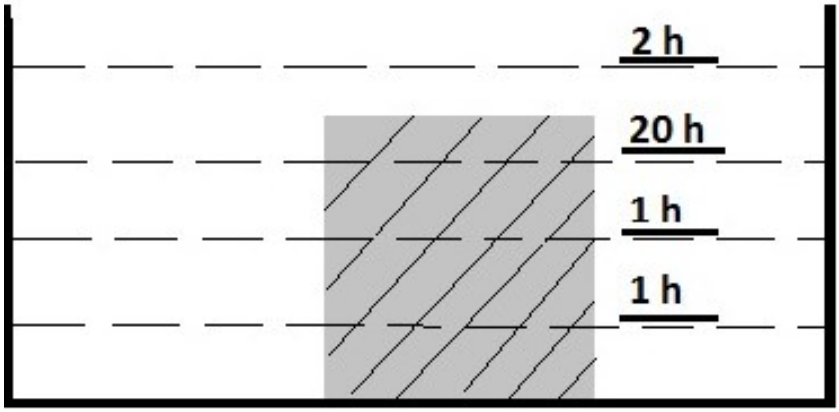

Figure 2. Scheme of gradual immersion of a sample in water

After each time interval, the samples were surface wiped with a sponge and their wet mass was measured. Based on determined sample masses in the dry and wet states, the water absorption values at different time periods were calculated.

One sample from each series was used for determination of water absorption and the other two samples for the determination of the compressive strength.

3. RESULTS AND DISCUSION

For design and commercial use of a concrete, certain characteristics have to be specified, such as: durability, strength, workability, density, production price and the like [20]. In recent years, special attention has been paid to those environmentally friendly materials, which are produced with minimal consumption of energy and resources, and preferably based on natural or recycled materials [21].

Hydraulic lime can be used as a binder to stabilize miscanthus creating a mineralized membrane, protecting it from the possibility of decay, inflammation or attack insects and rodents.

Metakaolin is a heat-treated (calcined) kaolinite ($Al_2Si_2O_5(OH)_4$) with high pozzolanic activity that leads to significant improvements in pore structure and thus increases the durability of concrete and resistance to harmful effects chemical and mechanical agents [28]. This contributes to lower consumption of conventionally the most used materials (cement and concrete) in construction materials. Because of that characteristics we choose this material for making samples for research.

After seven days of drying in the molds, the molds were disassembled and the samples were left to dry for another seven days (14 days in total) (Fig. 3), after which the characterization tests were performed [19].

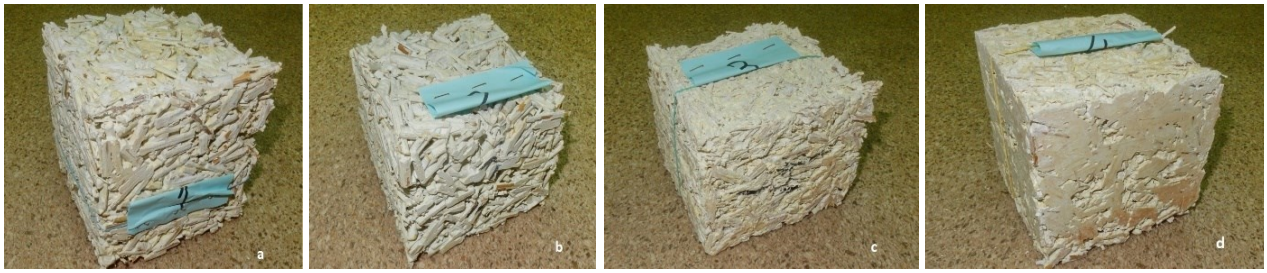

Figure 3. Samples after drying for 14 days obtained in four experimental series: a) 1, b) 2, c) 3, d) 4

Dimensions, mass, density, and compressive strength of dried samples are presented in Table 2.

Table 2. Dimensions, mass, density (γ_z), the force at which the sample cracks (P) and compressive strength ($f_{p,14}$) of dried samples from four experimental series

Series	*a* / mm	*b* / mm	c / mm	m_{14} / g	γ_z / kg m^{-3}	*P* / kN	$F_{p,14}$ / MPa
I	150.0	149.0	148,.0	13857	419	16.8	0.75
II	148.5	147.0	117.0	1184.7	464	18.5	0.82
III	149.5	147.0	107.5	1547.1	653	35.4	1.57
IV	149.5	151.0	152.5	2410.5	700	56.5	2.51

Samples in the first two experimental series were intensively crumbling at higher finger pressures, which is explained by the lack of binding components, while the samples in the III series were partially more compact. Samples in the IV series showed the best compactness and the highest density, which, in addition to the increase in the content of binders in the sample, can be also explained by the additional compaction of the material during placement in the mold. Both III and IV series were made without addition of Portland cement, so that it can be concluded that conventional Portland cement can be replaced by hydrated lime in these materials.

By the analysis of the results obtained for samples in II and III series, it can be concluded that the density increases with the increase in the fraction of binder components. In the examined experimental series, the density values range from 400 to 700 kg m^{-3} (Table 2), which is below the lower limit prescribed for concrete with brick admixtures in the interval between 800 and 1700 kg m^{-3} according to the Rulebook on energy efficiency of buildings of the Republic of Serbia [22]. Thus, based on the classification of concrete according to the standard SRPS EN 206, the tested materials have lower densities than those of lightweight concrete (800 to 1000 kg/m^3), but comparable to those of other, similar types of concrete with a filler of organic origin, for example a wood concrete (so-called "durisol"), which density values range from 550 to 800 kg m^{-3}.

As expected, samples in the IV series showed the highest compressive strength, *i.e*. 2.51 MPa was required to crack the sample (Table 2). The order of magnitude of the strength of the samples in series III and IV is in line with the strength of "durisol" materials ranging from 1 to 2.7 MPa [22]. Higher fineness of miscanthus chips, more binders and additional compactness in the mold, resulted in the production of a sample with the best characteristics.

The obtained results also correspond to literature values reported for the use of miscanthus in construction materials, where densities are ranging from 650 – 1250 kg m^{-3} and compressive strengths exceeded 2.5 MPa [13].

Examination of water absorption in the samples, showed high absorption values, as expected, ranging from 10 %, for sample immersion up to ¼ of the height, up to 57 %, when fully immersed. The percentage of water absorption was calculated based on measured sample masses before immersion in water, as well as after each repeated partial immersion and retention in water (Fig. 4).

Changing the granulometric composition and additional compaction of the material in the mold had a positive effect on the absorption of water of the samples, so that samples in the IV series had lower absorption values than samples from the I series.

All samples have more pronounced water absorption in the initial period, up to 2 h, after which the rate of absorption decreases. Samples from II and IV series have approximately the same values and trends of water absorption, which could be the result of a denser structure of these sample types.

According to previous research studies [23-26], some possibilities for the use of this miscanthus type in non-structural thermal insulation materials were also examined. In these studies, slaked lime (lime paste), electrostatic precipitator ash, and zeolite were used as binders. The results showed that such compositions containing miscanthus belong to the group of materials with good thermal insulation performances [27].

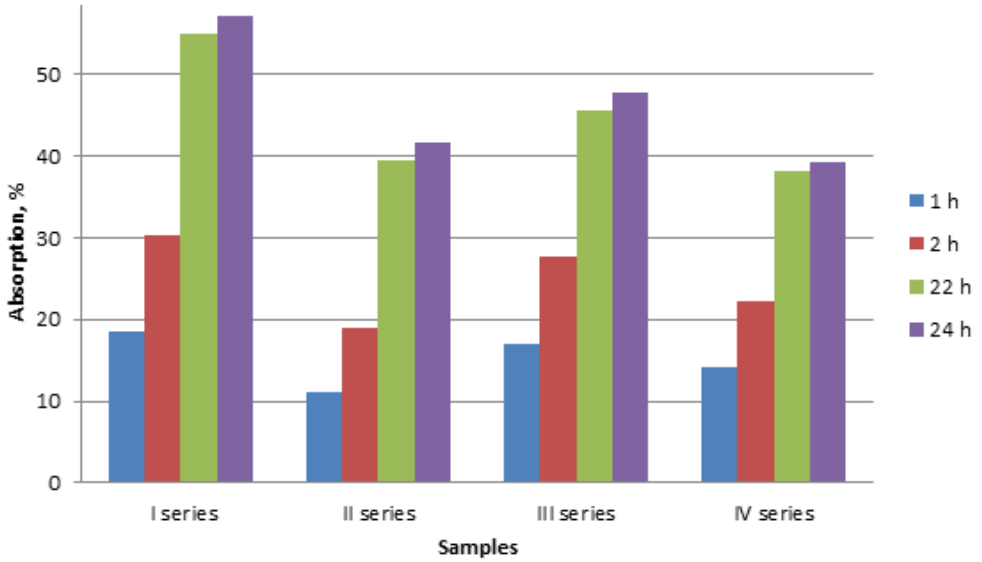

Figure 4. Water absorption values of samples from four experimental series at different times determined by the gradual immersion method

3. CONCLUSION

Mischantus x *giganteus* is a species which cultivation does not require significant financial costs, as it thrives on different types of land, adapts to environmental conditions, creates additional habitats for species, and significantly contributes to reducing CO_2 emissions from the production of building materials of this type,

The aim of this research was based on the use of this plant for production of lightweight concrete (bio-concrete) by substitution of conventional aggregates. This concept is in line with the principles of green low carbon economy and resource sustainability.

This phase of research has shown that the characteristics of lightweight concrete of this type are significantly influenced by the amount of binder, quantity, and fragmentation of aggregates (miscanthus), and especially the method of placement in molds so that at higher compression, higher values of the compressive strength and density are obtained. Based on the obtained results, the following conclusions can be drawn.

- The type of binder, the proportion of the binder component, the degree of fragmentation of the material and the method of installation in the molds have an impact on the tested properties of this lightweight concrete. The best results were obtained for the sample from series No. 4, which is composed of miscanthus, hydratated lime, metakaolin and gypsum, with the addition of water while the mixture was compressed into the mold.
- Sample from series No. 4 is characterized by the highest density, the lowest water absorption, and the highest compressive strength, which is the result of a thickened structure - the effect of packing smaller pieces of miscanthus and additional compaction of concrete in the mold. Samples of I series of the same composition containing 25 wt% miscanthus as in the IV series, but without additional compaction, have shown poorer characteristics, indicating that the key effect is additional compaction.
- Values of the measured properties: density and compressive strength of this lightweight concrete are in the range of properties of Durisol, a commercially used types of lightweight concrete.

By adding miscanthus to materials in the form of aggregates, we reduce the negative impact on the environment that has the production of conventional cement building materials. Given the favorable initial results of the research presented in this paper, this method of lightweight concrete production needs to be further studied, improved, and adapted to the market, which represents directions for further work.

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SAŽETAK

Miscanthus x giganteus za potencijalnu primenu u lakim betonima u građevinarstvu

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(Stručni rad)

Višegodišnja biljka *Miscanthus* x *giganteus,* iako potiče iz Azije, svoje stanište i višestruku primenu pronašla je i u Evropi. U ovom radu je prikazana potencijalna primena ove biljke u novim lakim građevinskim materijalima tzv. bio-betonima. Nadzemni deo biljke, nakon žetve, sušenja i usitnjavanja, je mešan sa vezivnim komponentama u različitim odnosima. Posle kalupljenja i sušenja, uzorci su karakterisani u pogledu fizičko-mehaničkih svojstava. Uzorak sa najvećim udelom veziva i sitnijom frakciom miskantusa, uz dodatno sabijanje u kalupe prilikom izlivanja, pokazao je najbolje karakteristike gustine i pritisne čvrstoće. Gustina uzorka je bila reda veličine kao vrednosti drugih vrsta lakih betona sa organskom ispunom, na primer betona sa ispunom od drveta („Durisol"), što dodatno opravdava korišćenje miskantusa u ove svrhe.

Ključne reči: bio-beton, biomasa, lignocelulozna vlakna, usevi, zelena gradnja, agro-energetski usevi

Surface modification method of duplex type stainless steels by the pack boriding process

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Abstract

This work presents the investigation of a boriding process on two grades of stainless steel namely UNS32750 super duplex stainless steel and UNSS31803 duplex stainless steel in order to improve material properties and possibly to reduce catastrophic failure of industrial components. Usage of duplex stainless steels has become customary in the fields of oil and refinery, marine and pipeline applications due to increased corrosion resistance; however, these materials exhibit low wear characteristics. To overcome this problem, in this work the pack boriding process was employed. Evaluation of effects of the boriding process on the microstructure and mechanical properties was performed using scanning electron and optical microscopy, Vickers hardness tests and wear tests. It was shown that the 4 h process resulted in the greatest boriding layer thickness yielding the maximum surface hardness of 1407 HV in the super duplex stainless steel UNS32750 while this value was 1201 HV in the duplex stainless steel UNSS31803. Wear resistance of borided materials were up to 6-fold greater than those of non – treated materials. Also, the borided duplex materials were shown to be more suitable for industrial applications for valve and shaft components as compared to the boronized super duplex stainless steel.

Keywords: UNS32750; UNSS31803; SEM; wear; hardness.

Available on-line at the Journal web address: http://www.ache.org.rs/HI/

TECHNICAL PAPER

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1. INTRODUCTION

Tribological properties of the duplex stainless steel family limit the usage of these metallic materials in industrial applications. Although these steels exhibit good corrosion resistance and mechanical strength, the wear resistance characteristics are poor. However, in hostile environments duplex stainless steels are the only alternative for standard austenitic stainless steels such as SS 304 and SS 316. Good strength and corrosion resistance of duplex stainless steels are due to the presence of chromium, nickel and nitrogen [1]. The increased mechanical strength, toughness and corrosion resistance makes these steels suitable in chloride environments [2]. These duplex and super duplex stainless steels, having increased pitting resistance [2] are applicable in the fields of oil and gas industry, marine applications, piping construction, chemical industry, and petrochemical plants [3]. Greater weldability and better mechanical properties of these steels as compared to those of austenitic stainless steels favor their usage in oil and gas refinery and chemical plants [4]. To increase the life of the sliding or mating parts, the material wear characteristics are considered as a dominant factor [5]. From the literatures, it is apparent that the valves and flanges require good tribological properties in addition to the corrosion resistance, which is not found in most of the materials. Good method to improve the material wear characteristics is by surface treatment. Among many surface treatment methods such as nitriding, carburizing, and nitrocarburizing, the boriding process based on the chemical treatment of the surface, was shown to

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result in increased hardness [6]. This process is not widely applicable in many industries unlike the conventional processes, even though it has many advantages likeproducing materials with high wear resistance, high galling property and good corrosion resistance [7]. The materials are heat treated with the boronizing medium at high temperatures to form extremely hard borided layers on the material surface [8]. Boron particles located on the boundaries between the grains, strengthen the bond between the boundaries and decrease the fragility [9]. The dual phase layer FeB and Fe_2B are formed on the outer surface and the growth kinetics are investigated by the analysis of the penetration depth of FeB and Fe_2B [10]. For low alloy steels the formation of FeB and Fe_2B increases the thickness of the boride layer as compared to that in high alloy steels because of the affinity of boron atoms for iron [6]. The FeB layer is harder than the Fe_2B layer, which promotes brittleness and surface defects on the material [11]. To overcome the difficulties of increasing the layer thickness in high alloy materials, the process temperature and time have to be increased [12-14]. The super duplex stainless steels contain several alloying elements such as chromium, nickel, manganese and molybdenum, which also reacts with boron and reduce the thickness of the iron boride layer [15]. Effects of the boronizing method on wear characteristics and the friction behavior were investigated on the Din 20 $MoCr_4$ steel material showing the hardness of the formed boride layer in the range 1475 to 1848 $HV_{0.05}$, while FeB and Fe_2B were observed on the surface of the material [16]. Many dual process treatments were also attempted like plasma nitriding, nitroboronizing, alumina boronizing and boronitrocarburizing [17]. Campos Silva *et al.* [18] studied the scratch and adhesion properties of the nickel boride layer on Inconel 718 super alloy formed by the powder-pack boriding process carried out at 900 ^oC for 2 to 6 h. The same process was applied in another study on a TB2 alloy and a diffusion model was proposed for the growth of the boride layer thickness [19]. A boronizing process was applied on the materials AISI 420 and 5120 resulting in approximately 5-fold lower wear rate as compared to that for theun-borided materials [20]. A solid boriding thermo-chemical treatment by using two boriding agents was applied on AISI H13 steel resulting in improved wear resistance as revealed by pin on disc tests [21]. Sista *et al.* [22] suggested that electrochemical boriding is an ultra-fast technique applicable for Inconel alloys to produce hard and protective layers on the material surfaces. From the literature, it is observed that duplex stainless steels have wide applications in the construction of pumps, shafts and valves, pressure vessels for petroleum, as well as in oil refining industrial processes and paper industry; however, for these applications high wear resistance is required [2]. Therefore, in this work we have performed experimental investigations of using a pack boriding process on UNS32750 and UNSS31803 stainless steels to improve the material mechanical and metallurgical properties.

2. EXPERIMENTAL METHODS

2. 1. Materials selection

The materials selected for the proposed work are super duplex stainless steel and duplex stainless steel (UNS32750 and UNSS31803), which were procured from M/s Jagruthi metal industries, Mumbai, India with the aim to investigate possibilities for improvements of the material characteristics by applying a boriding process. The standard chemical compositions with mass fraction ranges of alloy elements for the selected materials are given in Table 1.

Table 1. Chemical composition of super duplex UNS32750 and duplex UNSS31803 stainless steels [23]

Material	Content, wt.%									
	C	Mn	Si	P	S	Cr	Mo	Ni	N	Cu
UNS32750	0.03*	1.2*	0.8*	0.035*	0.02*	24-26	3-5	6-8	0.24-0.32	0.5*
UNSS31803	0.03*	2.0	1.0	0.03*	0.02*	21-23	2.5-3.5	4.5-6.5	0.08-0.2	-

*maximum

The boronizing medium consists of 90 % B_4C powder, having 325 mesh size, added with the diluent silicon carbide at 5 % and activator KFB_4 at 5 % (all chemicals procured from Alpha Aesar- Thermo Fisher Scientific, Mumbai, India). The SEM image of the boronizing medium is show in the Figure 1.

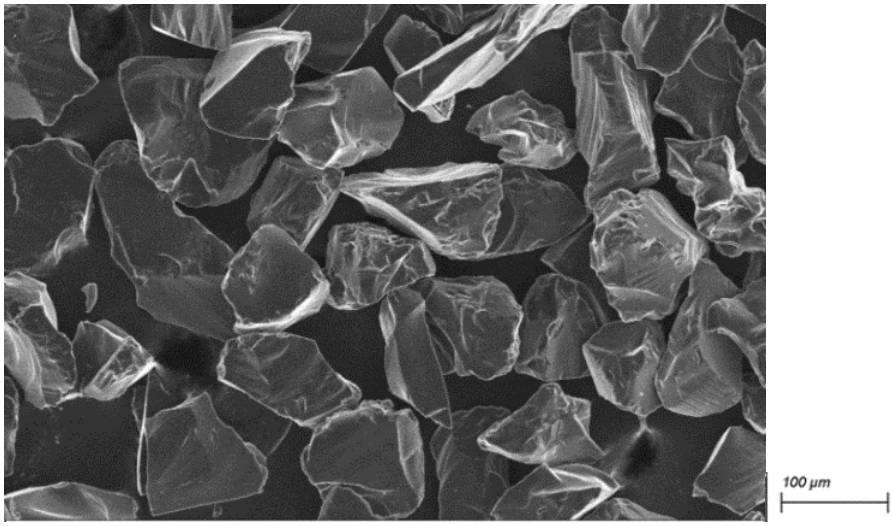

Figure 1. SEM image of the boriding medium

2. 2. Preparation of samples

Before the boriding process, surface grinding was carried out to the specification of 10 mm thickness to achieve the surface roughness of below 1.5 µm to increase the affinity for borondiffusion. The prepared cylindrical samples (ϕ 55 mm and 10 mm thick) were cleaned by acetone washing and individually kept in a SS304 container, covered with 3 mm of the boriding medium. Considering the environmental friendliness, ease of operation and low cost, the solid powder pack boriding process is used instead of toxic liquid and gas boronizing processes [24].

The workflow is briefly given as flowchart in Figure 2.

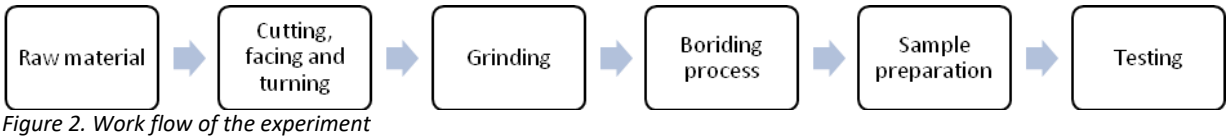

Figure 2. Work flow of the experiment

2. 3. Boronizing process

The electric furnace (Indfurr, Chennai, India) with specifications of 4.5 kW power and 1200 °C temperature was used for the boriding process. Initially a trial run was performed in order to select the process parameters. Based on the literature, the suggested temperatures for duplex stainless steel types are above 950 °C [25]. It was also inferred in the trial that at 1075 °C and the process duration of 5 h scales are formed due to formation of brittle FeB. Hence based on the industrial experts guidance the process parameters for the duplex stainless steels were fixed at the temperature of 1050 °C with varying process time of 2, 3 and 4 h. For each test three replicate experiments were performed.

2. 4. Testing methods

This investigation fulfils standards for metallographic analyses including determination of the microstructure, boride layer thickness, wear characteristics, hardness and surface roughness. In specific, scanning electron microscopy (SEM), optical microscopy, energy dispersive spectrometry, Vickers hardness tests, surface roughness tests, wear tests, and spark emission spectrography were used to determine metallurgical and mechanical characteristics of the borided samples. The surface roughness values were measured before and after boriding with the help of a surface roughness tester TR110 (Time Group, China).

An important factor considered for improving the wear resistance is the surface hardness which shows the capability to resist indentation. With the increase in hardness wear and corrosion resistances are proportionally improved. Hardness was determined by a Vickers micro hardness tester (CL/ME/MVIC40 with the FSA model, India). The samples are prepared in a bakelite mould process and the molded samples are polished to various grit sizes and finally diamond

polished for achieving a suitable surface finish. By using the Vickers micro hardness tester, the hardness values are determined on the surface, outer boriding zone, transition zone and the core with the indentation force of 50N.

By following the standard of ASTM E03-01 and the Metals Handbook [26] microstructure was analyzed by using an optical microscope with image analysis, (MVMS1310 Metavis, Medimage Technologies, India) at 25.3 °C. The etchant used in the mould was the mix of picric acid (1 g; 2,4,6 –trinitrophenol, Sisco Research Laboratory, India), HCL (5 cm^3; Sisco Research Laboratory, India) and ethanol (100 cm^3; Sisco Research Laboratory, India)

A drum type wear testing machine (Profilic Engineers, India) was used to compare wear characteristics of the materials by the weight loss method. The total revolution was 84 times, and the rotational frequency was 40 ± 1 rpm as per the industrial standard. The load applied on the material was 9.807 N (*i.e*. 1 kgf) and 60 grade coarse abrasive sheets were used. Initially before the wear test the sample was prepared was cut in a circle with the specified diameter of 15 mm and the thickness of 10 mm. The weight was measured before and after the wear test for both investigated materials. The difference between the initial and final weights is considered as the abrasion loss. For each test three replicate measurements were carried out and the average values were noted.

The samples for SEM analysis were cut into 8 mm squares by a wire cut EDM machine. By using various grit sizes of emery sheets the samples were carefully mirror polished and cleaned using acetone. The etchant was then applied on the samples and the cut section morphology was analyzed by using field emission scanning electron microscopy (SIGMA with Gemini column, Carl Zeiss, USA). The elemental composition of the materials was analyzed by energy dispersive spectroscopy (EDS) by using an EDAX analyzer (Nano XFlash Detector, Bruker, Germany)

Composition of the materials wasexamined by preparing samples (ϕ10 mm) for spark emission spectrography (OBLF, Germany). The spark emission test was taken at one spot.

3. RESULTS AND DISCUSSIONS

3. 1. Chemical composition

Chemical composition of both stating materials was determined by spark emission spectroscopy (Table 2) confirming the specifications.

Table 2. Chemical composition of super duplex UNS32750and duplex UNSS31803 stainless steels used in the present study

	Content, wt.%								
Material	C	Mn	Si	P	S	Cr	Mo	Ni	Nb
UNS32750	0.018	0.70	0.52	0.02	0.009	24.36	3.86	6.34	0.269
UNSS31803	0.015	1.35	0.8	0.017	0.012	21.74	2.83	4.59	0.112

3. 2. Surface roughness

Surface roughness is an important material characteristic, and it is altered by diffusion of the boriding medium.

Therefore, the average surface roughness values (R_a) were measured before boriding to find the extent of improvement in R_a values after the process. Decreased surface roughness after boriding was also reported in literature [12]. The measured values presented in Table 3, indicate that the boriding process slightly smoothens the rough surface due to the diffusion of boron.

Table 3. Surface roughness values

Material	Boriding duration, h	R_a / μm (before boriding)	R_a / μm (after boriding)
super duplex stainless steel UNS32750	2	0.71 ± 0.08	0.59 ± 0.06
	3	0.66 ± 0.06	0.56 ± 0.04
	4	0.82 ± 0.06	0.58 ± 0.06
duplex stainless steel UNSS31803	2	0.93 ± 0.08	0.68 ± 0.03
	3	0.63 ± 0.05	0.48 ± 0.04
	4	0.56 ± 0.04	0.5 ± 0.03

3. 3. Hardness

Average hardness values based on 3 readings from each area are presented in Figure 3. Hardness values of the outer boride layer, transition zone and core are measured in the molded sample and the surface hardness is measured at the boronized surface. All of the three samples (*i.e.* boriding for 2, 3 and 4 h) for both materials were analyzed. Hardness in the core area of super duplex UNS32750 and duplex UNSS31803 stainless steels is around 290 HV and 240 HV, respectively.

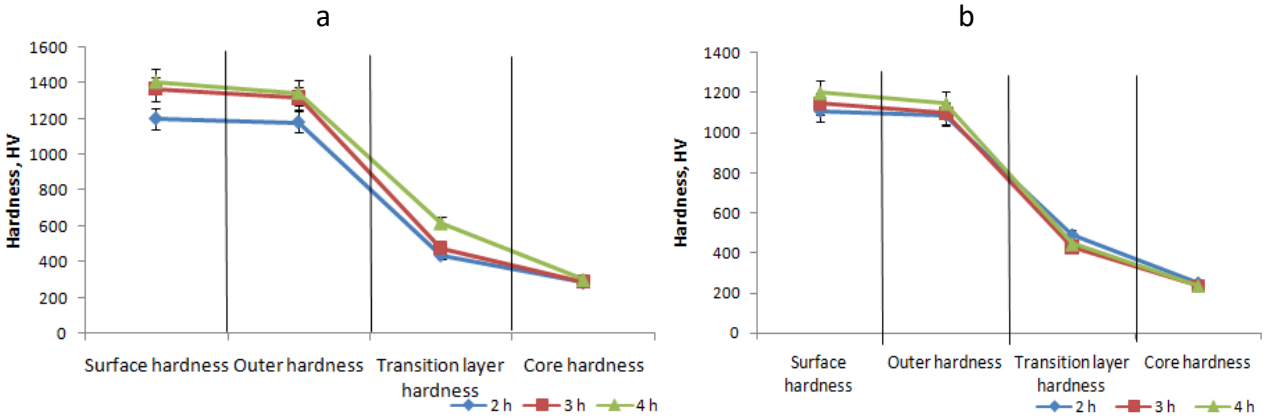

Figure 3. Surface hardness and case depth for (a) super duplex stainless steel UNS32750 and (b) duplex stainless steel UNSS31803

Hardness values for both investigated materials improved drastically. The outer surface shows the maximum hardness level, followed by the boriding layer and transition zone. The higher hardness of the core in the super duplex stainless steel UNS32750 than in the duplex stainless steel UNSS31803 may be due to the presence of more chromium in addition to nickel and nitrogen.

From the obtained results it is inferred that the hardness at the surface, outer and transition zone is increasing over time of boriding. The increase in hardness was reported with the increase in temperature and time [24]. This may happen due to longer time for a boron particle to diffuse to the base metal which increased the thickness of the boriding layer. As a result, the surface hardness increased 5-fold in the duplex stainless steel UNSS31803 and 4.5-fold in the super duplex stainless steel UNS32750 as compared to the base material hardness. This will produce effects on the wear and corrosion resistance.

The decreasing hardness trend in various zones as observed in Figure 3 may be due to the boron diffusion. Longer time for diffusion leads to formation of dual phase of FeB and Fe_2B on the surface and the boronizing zone. The FeB is harder and becomes brittle on the surface and followed with the Fe_2B phase. Diffusion is lower in the transition zone as compared to that on the surface. Boron diffusion practically does not take place in the core area which therefore exhibits the same hardness as the base material. Thus, Figure 3 clearly shows the increased surface hardness of both materials as compared to that of the base materials.

3. 5. Boride layer thickness and metallographic evaluation

Thickness of the boride and transition layers was determined by optical microscopy as shown in Figure 4(a). The interface layer is the transition zone between the boriding, and the base microstructure as shown in the micrograph in Figure 4(b). The case depth increases as the boriding time is increased. The boriding layer thickness in the duplex stainless steel UNSS31803 is slightly greater than that in the super duplex stainless steel UNS32750 due to lower amounts of alloying elements in the former case.

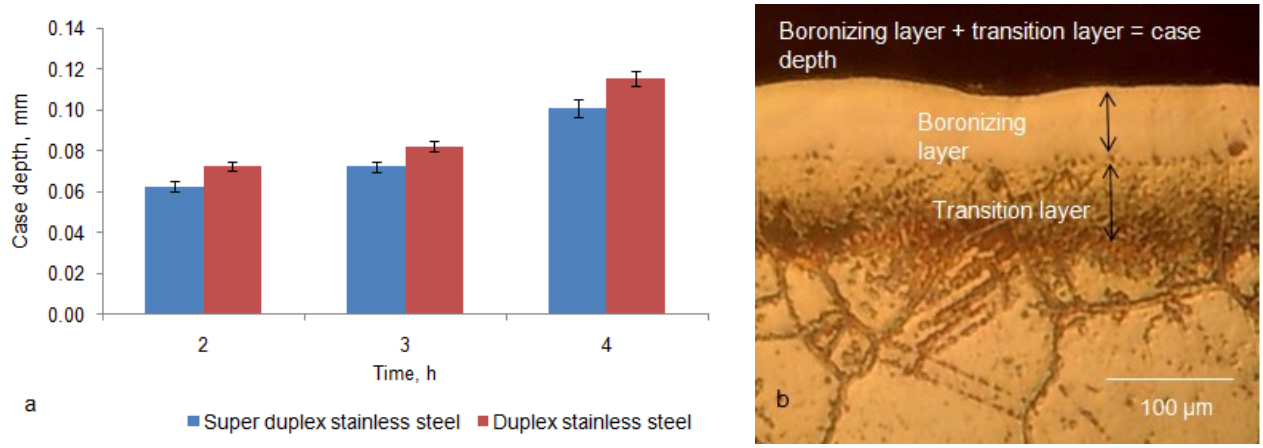

Figure 4. (a) Case depth as a function of boriding time; (b) representative optical micrograph on the right shows the boriding and interface layers on the cross-section of one sample

The microstructural analysis was carried for all samples while SEM images and EDX analysis are presented in Figure 5 for materials exposed to boriding for 4 h.

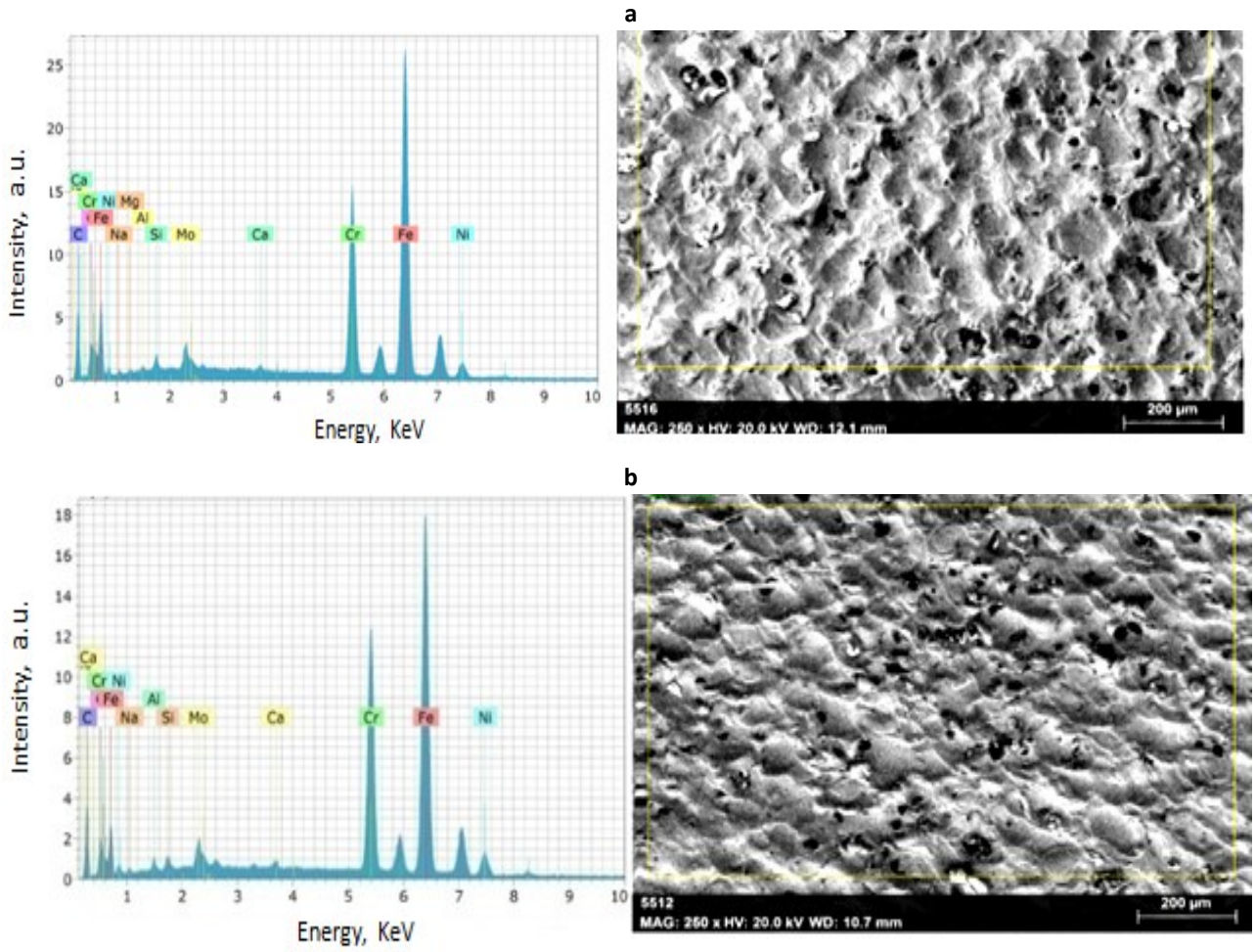

Figure 5. SEM micrographs and EDX analyses for materials exposed to the 4 h boriding process for: (a) duplex stainless steel UNSS31803, (b) super duplex stainless steel UNS32750; (black dots in SEM micrographs correspond to surface pores; scale bar: 200 µm)

It can be observed that low porosity appears on the surface of the materials as revealed by black dots on the surfaces (Figure 5). The porosity is lower in the duplex stainless steel UNSS31803 as compared to the super duplex stainless steel UNS32750 due to lower amounts of alloying elements in the former case.

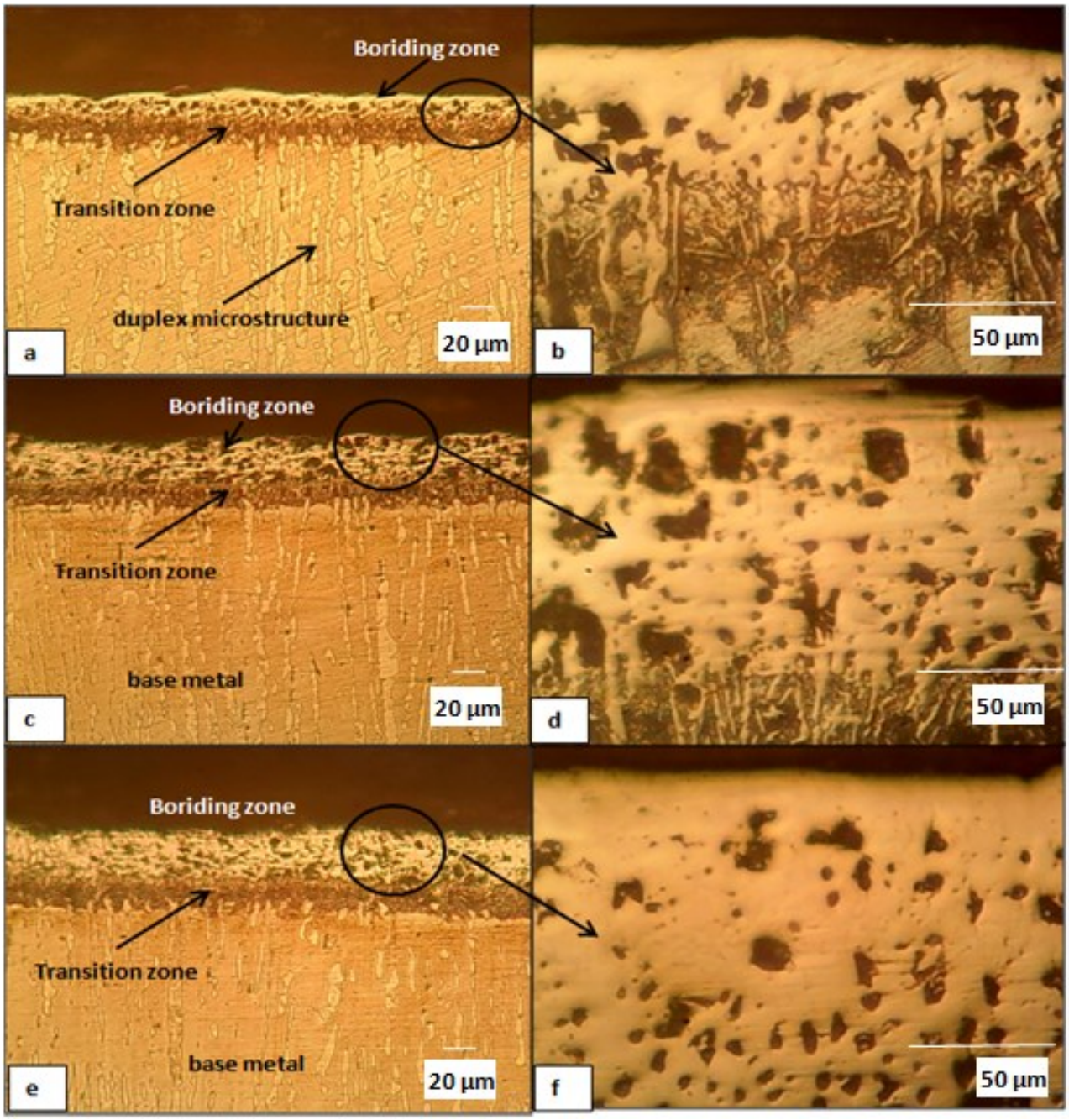

Figure 6. Optical microscopic images showing microstructure of duplex stainless steel UNSS31803 exposed to boriding for: a, b) 2 h; c), d) 3 h; e, f) 4 h

Figure 6 shows microstructures of the UNSS31803 material exposed to boriding for 2, 3 and 4 h. The microstructure of the base metal clearly shows the duplex stainless steel. The total dispersion of boron is the combined layer thickness of boriding layer and transition layer. For 2 h boriding on the surface of the base metal the total dispersion of boron layer is 73 µm, in which the boriding layer is about 36 µm thick and the transition zone is formed ~38 µm thick below the boriding layer. (Figure 6a, b) The transition layer indicates the saw tooth morphology, which is commonly seen as

columnar like structure after the boriding layer. With the increase in process duration to 3 h the thickness of boriding and transition layers is measured as ~47 μm and ~35 μm, respectively as shown in Figure 6 c, d.

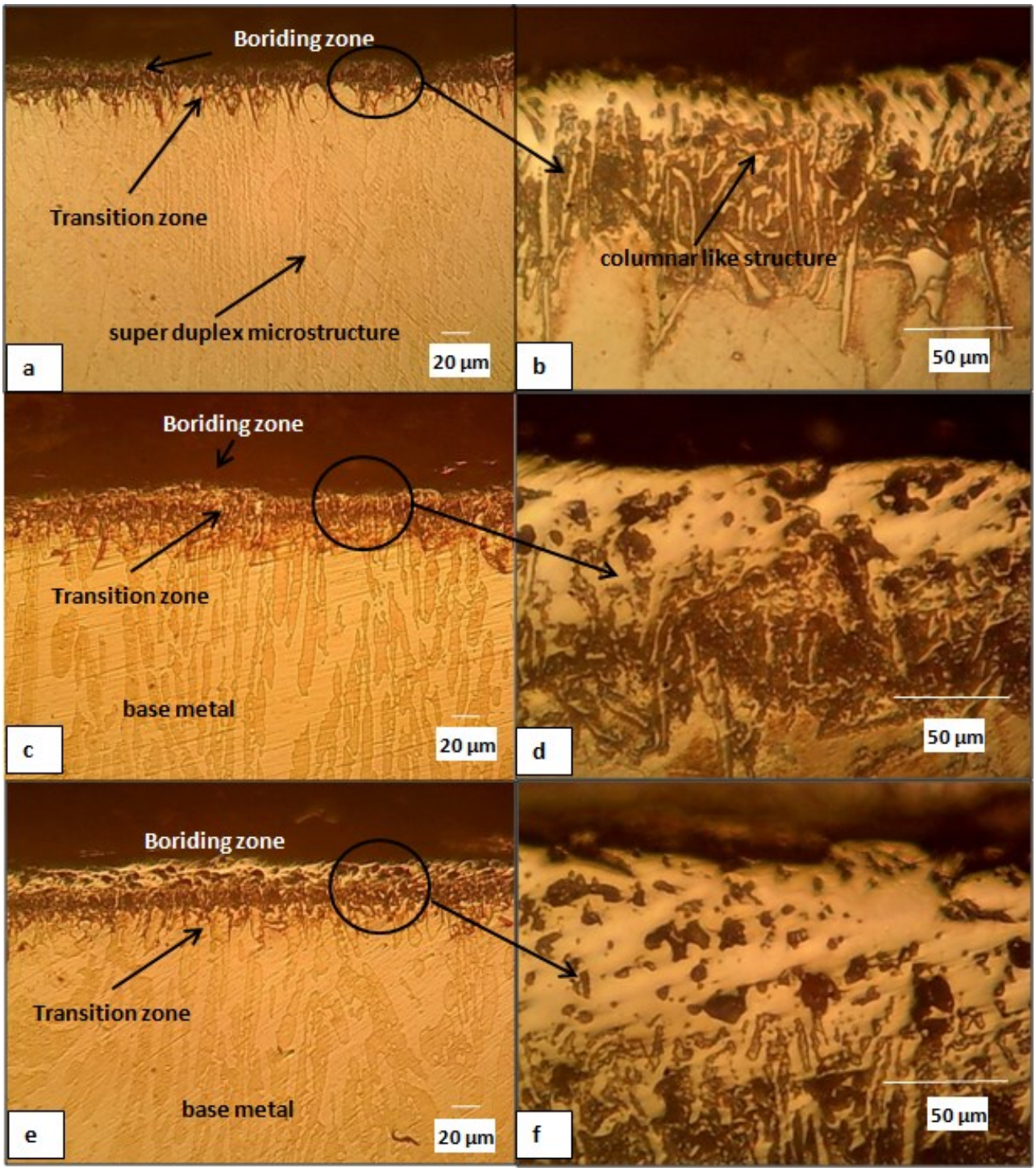

Figure 7. Optical microscopic images showing microstructure of super duplex stainless steel UNS32750 exposed to boriding for: a, b) 2 h; c, d) 3 h; e, f) 4 h

Further increase in boriding duration to 4 h results in increased thicknesses of both boriding and transition layers of around 116 μm shown in Figure 6 e, f. Due to the extension of time the boronizing medium have more time for better diffusion and the layer thickness is more compared to the 2 h and 3 h process.

Boriding of the super duplex stainless steel UNS32750 for 2 h resulted in formation of a very thin boriding layer of 5 μm and transition layer thickness of ~58 μm (Figure 7 a, b). With the increase in the boriding duration the boriding layer thickness is increased. The super duplex microstructure is clearly seen in the base material. The uneven iron boride layer formation and low thickness may be due to the restriction of bonding between the iron and boron.

The other alloying elements shared the boron atoms due to the high contents such as NiB2, CrB_2. The saw tooth microstructure was observed in the transition layer. The layer thickness formed for 3 h process is ~72 μm and for 4 h process it is ~101 μm respectively which also shows the increase of time of boriding process increases the boriding layer thickness. Even though the temperature was kept constant at 925 °C, the boride layer thickness increases with the extension of time to 4 h.

3. 6. Wear test analysis

The removal or deformation of the material at the surface corresponds to the wear property of the material. There are many wear types such as adhesive, abrasive, and fretting wear, which damage the material and decrease the life of the products. Thus, to find the wear resistance, in this work the effects of abrasive wear was investigated. The abrasion loss was calculated from the difference between the final weight after the wear test and the initial weight before the test [27] and the results are shown in Figure 8.

The weight loss of untreated samples measured as 0.25 and 0.32 % for duplex UNSS31803 and super duplex UNS32750 stainless steels. The weight loss after boriding for 4 h for the duplex stainless steel UNSS31803 was only 0.04 % while for the super duplex stainless steel UNS32750 was 0.12 %. Both materials show significantly lower abrasive wear losses after the boriding process and hence proved that the wear resistance of the boronized samples is higher and that the life of the final products would be improved. In specific, the wear loss is ~6-fold and 2.5-fold lower for the borided duplex stainless steel UNSS31803 and the super duplex stainless steel UNS32750 samples, respectively, as compared to the corresponding values of non-treated samples.

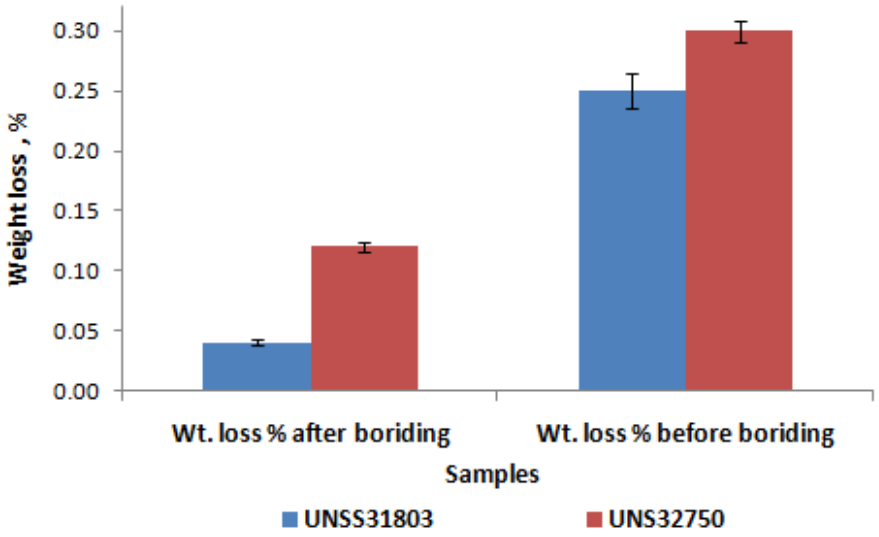

Figure 8. Weight loss comparison in the wear test

The samples submitted to the wear test were analyzed regarding the surface roughness in terms of Ra and Rz values. The average roughness R_a and R_z values for the super duplex stainless steel UNS32750 were ~6 μm and ~13 μm, respectively, while for the duplex stainless steel UNSS31803 these values were ~4 μm and 9 μm, respectively. Thus, surface roughness was lower of UNSS31803 samples as compared to that of UNS32750 samples.

The obtained results clearly indicate that the increased boride layer thickness in the duplex stainless steel UNSS31803 sample ensures lower surface roughness as compared to that in the super duplex stainless steel UNS32750

sample. The difference in average surface roughness (R_a) value was ~2 μm but R_z values differed by 4 μm between the two materials. It was suggested in literature that the variation was due to the formation of a thicker layer of FeB and differences in the composition of material substrates [28]. Due to higher diffusion of boron and a thicker boride layer in the UNSS31803 sample, the wear resistance increased, and the wear depth was reduced causing lower surface roughness than that of the UNS32750 sample.

4. CONCLUSION

Investigation of the boriding process on UNS32750 super duplex stainless steel and UNSS31803 duplex stainless steel samples was carried out with the aim to improve the wear resistance of these materials. The following observations were derived from the experimental analysis.

- In both investigated materials, the borided samples shows slight decrease of surface roughness values compared to the non treated samples. The results may be due to the diffusion of boron which smoothed the surface.
- Longer boron diffusion by extending the boriding process time increased the material hardness. The maximal value of the surface hardness of 1407 HV was achieved in the super duplex stainless steel UNS32750 sample after 4 h of boriding while this value for the duplex stainless steel UNSS31803 sample was 1201 HV. Measured hardness values decreased from the surface boride layer to the interface layer and further to the base material.
- Surface morphology of the samples after boriding showed uneven surfaces although the surface finish improved. Microstructures of both materials confirmed the duplex stainless steel type. Thicknesses of the boride and interface layers increased over time of boriding in both materials due to prolonged boron diffusion. After 4 h of boriding the total boron diffusion layer in the duplex stainless steel UNSS31803 was ~115 μm thick while it was ~100 μm thick in the super duplex stainless UNS32750. The prolonged boron diffusion increased the boride layer thickness and improved surface finish.
- Wear characteristics of boronized materials improved as compared to non-treated samples so that the improvement was ~2.5-fold for the super duplex stainless steel UNS32750 sample while even 6-fold for the duplex stainless steel UNSS31803 sample.
- From the results obtained in this experimental work it can be concluded that the duplex stainless steel UNSS31803 is more suitable for application of the boriding process with respect to the improvement in wear resistance. Still, the borided samples of both stainless steels are suitable for applications in pumps and valves in the oil and petroleum fields possibly preventing the irrecoverable losses by the improvement of surface properties. These borided materials could thus increase the life of products due to high wear resistance and good surface characteristics in addition to the higher corrosion resistance.

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SAŽETAK

Metoda modifikacije površine dupleks nerđajućih čelika postupkom boriranja

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Ovaj rad prikazije istraživanje procesa boriranja dve vrste nerđajućeg čelika: UNS 32750 super dupleks nerđajući čelik i UNSS 31803 dupleks nerđajući čelik, u cilju poboljšanja osobina materijala i smanjenja mogućnosti katastrofalnih otkaza industrijskih komponenata. Upotreba dupleks nerđajućih čelika postala je raširena u postrojanjima za vađenje nafte i rafinerijama, u pomorstvu i u cevovodima, a zbog njihove povećane otpornosti na koroziju. Međutim ovi materijali su pokazuju slabu otpornost na habanja. Da bi se prevazišao ovaj problem, u ovom radu je korišćen proces borirawa. Procena efekata boriranja na mikrostrukturu i mehanička svojstva izvršena je pomoću skenirajuće elektronske i optičke mikroskopije, testova tvrdoće po Vickersu i testova habanja. Pokazano je da je postupak boriranja u trajanju od 4 sata rezultirao najvećom debljinom boriranog sloja, dajući maksimalnu površinsku tvrdoću super dupleks nerđajućeg čelika UNS 32750 od 1407 HV, odnosno 1201 HV dupleks nerđajućeg čelika UNSS 31803. Otpornosti na habanja boriranih materijala bile su i do 6 puta veće od onih kod netrfetiranih čelika. Pokazalo se da su borirani dupleks materijali pogodniji za proces boriranja jer pokazuju veću otpornost na habanje u poređenju sa super dupleks nerđajućim čelikom, zbog povećane debljine sloja gvozdenog borida.

Ključne reči: UNS32750; UNSS31803; SEM; habanje; tvrdoća

Glazing effect for producing environmentally friendly ceramics for cladding applications

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Abstract

This paper presents results of comparative studies of environmental safety of ceramic materials, based on a low-plasticity clay with the introduction of galvanic sludge, boric acid and titanium dioxide in 3 different combinations. The experimental samples were manufactured under 15 MPa pressing pressure and at the maximum firing temperature of 1050 °C. Prior to the toxicological experiments, diurnal extracts of the materials into the model neutral and acidic media were obtained. The toxicological safety was determined by using the *Daphnia* mortality method, and by comparing the maximum permissible concentrations of heavy metals for drinking and household water with the heavy metals' concentrations in diurnal extracts. The presented data show that the combined introduction of all the investigated additives results in the glazing effect of ceramic particles surfaces so that an environmentally safe material can be produced that exhibits sufficiently high-performance properties. The use of low-plastic clay and electroplating sludge expands the raw material base for producing ceramics and allows the disposal of environmentally hazardous compounds of heavy metals contained in electroplating sludge. Ceramic materials based on the developed charge composition can be used for producing items for external cladding for buildings and structures.

Keywords: environmental safety; vitrification; low-plasticity clay; electroplating sludge; heavy metals.

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1. INTRODUCTION

Technologies for utilization of various waste types in production of construction materials are simple and provide disposal of considerable amounts of waste. Such waste utilization stimulates savings of primary natural resources, while simultaneously reducing the production cost and environmental pollution by waste [1-3].

However, in most cases, the use of waste reduces the quality and operational properties of the manufactured materials and products and is also associated with the necessity to ensure their environmental safety, since waste components and substances, formed during their disposal, often belong to fourth hazard class substances at best. In this regard, it is required not only to determine the waste content, ensuring the regulatory requirements compliance for the materials and products performance, but it is also necessary to provide the environmental safety assessment. Besides, in production of building materials waste is in most cases used only as a cheap filler, not utilizing all possible valuable properties of substances and component, contained in waste.

We have previously studied the use of various wastes as functional additives for improving the quality and performance properties of construction materials. In one of the studies, the combined introduction of electroplating sludge, boric acid and titanium dioxide provided production of ceramics with a self-glazing effect on the sample surfaces, which reduced water absorption and increased frost resistance [4].

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Recycling of electroplating sludge is up to date, since it is classified in 2-3 hazard waste classes and its processing by using other methods is time and resource consuming [5-7]. The application of the obtained composition is relatively simple and allows galvanic sludge utilization as a pore-forming agent and vitreous phase modifier.

The research objective was to study the impact of the vitrification effect, obtained during combined introduction of electroplating sludge, boric acid and titanium dioxide, on environmental safety of the resulting ceramics aimed for cladding applications.

2. MATERIALS AND METHODS

2. 1. Sample production

In this research we consider the ceramic material produced from the clay of Suvorotskoye Deposit in the Vladimir region (Russia) of the following composition (wt.%): 67.5 SiO_2; 10.75 Al_2O_3; 5.85 Fe_2O_3; 2.8 CaO; 1.7 MgO; 2.4 K_2O; 0.7 Na_2O [8,9]. The clay plasticity index was determined by the standard method as 5.2. Therefore, according to the standard GOST 9169-75, this clay belongs to the low-plasticity grade, so that the resulting manufactured items are low quality products *i.e*. of low strength and high water absorption. Thus, the effective application of this clay requires the introduction of modifying additives.

Boric acid (BA) grade B (ProfSnab LLC, Russia) 2nd class (GOST 18704-78) was used as the first additive, being a strong fluxing agent and providing liquid-phase sintering, which increases ceramics strength and reduces its water absorption [10].

The second additive was galvanic sludge (GS) formed as a result of reagent treatment of waste water at the Avtopribor plant (Vladimir, Russia), and is characterized by the following composition (wt.%): $Zn(OH)_2$ ≈ 11.3 %; SiO_2 ≈ 7.08 %; $Ca(OH)_2$ ≈ 16.52 %; $Cr(OH)_3$ ≈ 9.31 %; $(Fe^{2+})Cr_2S_4$ ≈ 4.17 %; $CaCO_3$ ≈40.25 %; CaO ≈ 3.45 %; ZnO ≈ 2.41 %; $Cu(OH)_2$ ≈ 2.38 %; $Ni(OH)_2$ ≈ 2.62 %; $Mn(OH)_2$ ≈ 0.64 %; $Pb(OH)_2$ ≈ 0.14 % [11].

In this study, titanium dioxide (TiO_2, NEO Chemical, Russia) of R-02 brand (GOST 9808-84) was considered as an additional additive affecting composition and structure of crystallization products and providing together with the other specified additives the vitrification effect in the depth of the samples.

Samples of the obtained material were made by using semi-dry pressing technology. The pre-crushed clay and electroplating sludge with the fraction of max 0.63 mm particle size were selected and dried to a constant mass. The choice of the fraction particle size, as well as the choice of subsequent parameters for manufacturing the ceramic samples, were based on the previous experiments in which the best results for mixing, compacting and sintering of ceramics, based on the used clay, were obtained at these parameters values. Next, all the charge components were initially mixed dry in accordance with the studied charge compositions, followed by mixing with water at the concentration of 8 wt.% in order to achieve uniform molding mass. The mixing period at each stage lasted 5 min. The ceramic samples were made from the molding mass by one-side pressing under the pressure of 15 MPa, followed by firing in the oxidizing atmosphere at the heating rate of 5 $^oC\ min^{-1}$ with the exposure at the temperature of 1050 ^oC during 30 min. The samples were made cube shaped of 50 mm side in three sample series for each charge composition, followed by averaging of the experimental results for each series.

2. 2. Characterization of the obtained ceramic samples

2. 2. 1. Compressive strength

The compressive strength was determined by continuous and uniform load application to the sample to reach its destruction with the maximum load fixation using hydraulic press P6326B (JSC "Gidropress", Russia).

2. 2. 2. Water absorption

Water absorption has been determined by the sample dry mass increase after saturation with water at the atmospheric pressure during 48 h.

2. 2. 3. Frost resistance

Frost resistance of the sample has been determined after the water absorption experiment. For this purpose the water-saturated sample has been kept during 4 h at the temperature in the range from -15 °C to -20 °C, and afterwards placed in water at room temperature for 2 h and examined for cracks. In the case that cracks were not found, a new freeze-thaw cycle was performed for the sample.

2. 3. Toxicological studies

Toxicological studies of the considered compositions containing additives were performed for the ceramic samples, using the biotesting method of *Daphnia magna Straus* mortality under the influence of the substances present in diurnal water extracts from the samples of the studied materials. To account for possible mechanical product damage and wear, the whole samples were slightly chipped and placed in 500 cm^3 of distilled water each for 24 h. Next, three samples (100 cm^3 each) were taken from the resulting extract. Separately, the control 100 cm^3 sample of the cultivation water was made. 10 daphnia from the pre-staged synchronized culture, grown in cultivation water were placed in each sample. The number of live and dead daphnia was counted once a day during 96 h. Daphnia were considered dead in the case of absence of movements within 15 s after a light shake of the glass with the sample. The experiment was considered reliable when at least 9 daphnia survived in the control sample.

To account for the likely effects of acid rain and soil acidity, chemical testing concerning the degree of heavy metals migration into the diurnal water (pH 7.2) and ammonium acetate (pH 4.8) from the samples of the studied materials has been performed. Zinc, chromium, copper, and nickel ions were chosen for assessment, since electroplating sludge contains the largest amount of these heavy metals' compounds. The extracts were prepared by placing the slightly chipped samples into 500 cm^3 of distilled water and 500 cm^3 of ammonium acetate solution, each. The acetate-ammonium buffer was prepared by adding 108 cm^3 of glacial acetic acid into 500-600 cm^3 of distilled water (ch. p, Lenreactive PLC, Russia) of 99.8 wt.% CH3COOH and 75 cm^3 of 25 % aqueous technical ammonia solution $NH_3 \cdot H_2O$ (brand A, PLC KuibyshevAzot, Russia). Quantitative detection of heavy metals in water extract from the samples has been performed by using an atomic-absorption spectrometer Quant-Z.ETA-T (NEO Chemical, Russia). The ceramics structure was studied by using Quanta 200 3D scanning electron microscope (FEI Company, USA).

3. RESULTS AND DISCUSSION

In this research the comparative studies of compositions not containing and containing the considered additives have been carried out (Table 1).

Table 1. Compositions and properties of the studied ceramic materials

№	Additive amount, wt.%)			Compressive strength, MPa	Water absorption, %	Frost resistance (cycles)
	GS	BA	TiO_2			
1	-	-	-	14.3±0.7	7.5±0.5	39±2.0
2	5	-	-	11.6±0.6	12.7±0.7	30±2.0
3	5	5	-	21.3±0.9	7.2±0.4	42±2.0
4	5	5	10	21.0±0.9	3.2±0.2	51±3.0

Toxicological studies have shown (Fig. 1) that the composition 2, containing only galvanic sludge, caused 100 % *Daphnia* mortality on the second day of the experiment, while the composition 3 containing galvanic sludge and boric acid caused *Daphnia* mortality not exceeding the critical value of 50 % throughout the experiment, although approaching it in 96 h.

Data concerning the composition without additives (composition 1) and the composition containing all the considered additives (composition 4) are not shown in Figure 1 as during 96 h all investigated planktonic organisms survived in the corresponding diurnal water extracts, indicating the material environmental safety.

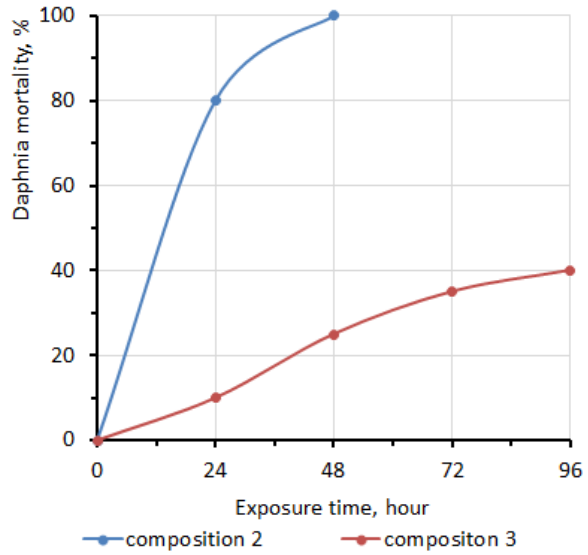

Figure 1. Dependence of Daphnia mortality on exposure time and sample composition

The obtained results can be explained by structural features of the studied ceramic materials. The initial ceramic material without any additives constitutes the sintered ceramic grains (Fig. 2a), while the galvanic sludge, introduced into the charge, caused the grain debonding and porosity increase (Fig. 2b), which is confirmed by the increase in water absorption (see Table 1).

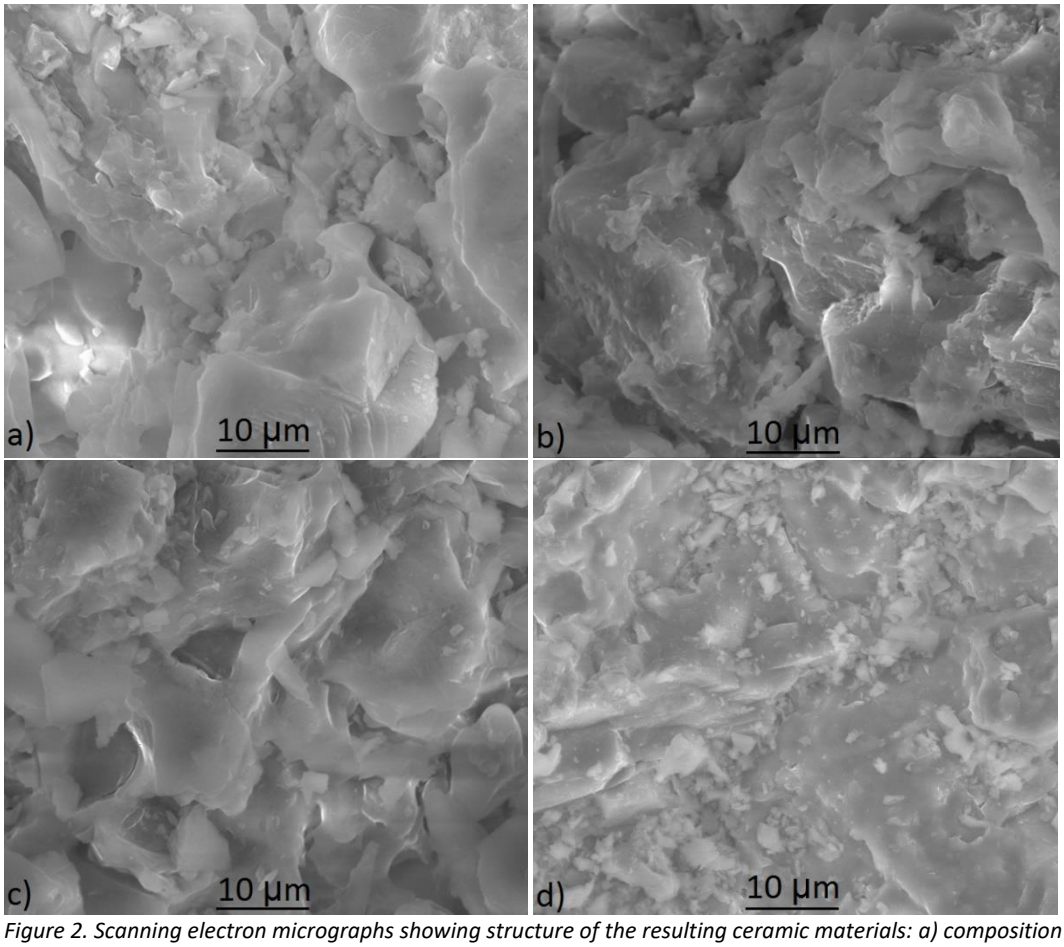

Figure 2. Scanning electron micrographs showing structure of the resulting ceramic materials: a) composition 1; b) composition 2; c) composition 3; d) composition 4

In addition to the porosity increase, the surface area of ceramic particles that is in contact with the aqueous medium also increases, which promotes heavy metal ions migration into the medium. Boric acid introduction along with the electroplating sludge causes vitreous phase formation in the ceramic structure (Fig. 2c), which partially fills pores and voids, thus reducing water absorption and heavy metal ions migration into the aqueous medium. When all three additives are used, the ceramic particles surface is vitrified, causing further increase in the vitreous phase content, covering most of the surfaces of ceramic particles with layers of different thicknesses and closing most of the open pores (Fig. 2d).

The extraction experiments (Table 2) demonstrated that samples containing electroplating sludge without the additional additives (composition 2), release the highest concentrations of all the considered metals in acidic media as compared to the other compositions. These concentrations are above the limits in the acidic extracts while although lower in the neutral medium (*i.e*. distilled water) still above the limits for chromium and nickel. The obtained concentrations , considering standard deviations from the average values, were compared to the maximum permissible concentrations (MPC) in water bodies used for domestic, drinking and recreational purposes [12].

Table 2. Determination of heavy metals concentrations in test media

Metal	Metal concentration, mg dm^{-3}		Metals MPC in water bodies*, mg dm^{-3} [12]
	In distilled water extract (pH 7.2)	In ammonium acetate extracts (pH 4.8)	
		Composition 2	
Zinc	0.324±0.016	1.895±0.095	1
Chromium	0.058±0.003	0.295±0.015	0.05 for Cr^{3+}
Copper	0.012±0.001	1.514±0.076	1
Nickel	0.046±0.002	0.994±0.05	0.02
		Composition 3	
Zinc	0.050±0.003	0.292±0.015	1
Chromium	0.009±0.001	0.045±0.002	0.05 for Cr^{3+}
Copper	0.002±0.001	0.233±0.012	1
Nickel	0.007±0.001	0.153±0.008	0.02
		Composition 4	
Zinc	0.011±0.7	0.066±0.003	1
Chromium	0.002±0.001	0.01±0.001	0.05 for Cr^{3+}
Copper	0.000±0.001	0.052±0.003	1
Nickel	0.002±0.001	0.014±0.001	0.02

*used for household, drinking and recreating purposes

The excess of nickel ions in acidic media is observed for the samples produced with the combined introduction of galvanic sludge and boric acid (composition 3), while in neutral media, *i.e*. under normal operating conditions, the material can be considered environmentally safe. The normal operating conditions for this material include standard weather conditions in contact with the materials, typical for temperate climate latitudes in cold and warm seasons, in case the products are for exterior usage for buildings and structures cladding. Non-standard operating conditions when the material is in contact with low-pH environments may include acid rain, soil and aggressive chemicals. According to the data presented in the table, such material (composition 3) can only be used for standard operating conditions. The heavy metal ions concentration does not exceed the maximum concentrations in both neutral and acidic environments, *i.e*., both are suitable for standard and non-standard operating conditions for the samples produced using all the considered additives (composition 4). In this case, environmental safety is ensured by the vitrification effect, created by the combined application of all additives: boric acid is the fluxing agent, titanium dioxide is the vitreous phase source, and galvanic sludge is the modifier of vitreous phase properties. Herewith most of heavy metal compounds are a part of the vitreous phase, migration from which is almost impossible at normal performance conditions and is insignificant in acidic media.

4. CONCLUSION

Based on the conducted research results it can be concluded that by the sole galvanic sludge introduction, it is impossible to produce an ecologically safe material. But in the case that both galvanic sludge and boric acid are used, environmentally friendly construction materials can be produced only for applications under standard conditions.

However, under the exposure in acidic media, migration of environmentally hazardous heavy metals ions from this material is potentially possible. It means that this material cannot be used for the constructional cladding.

At the same time, a combined introduction of electroplating sludge, boric acid and titanium dioxide provides the development of environmentally safe ceramic cladding materials that can be used in any environmental conditions. The resulting ceramics possessing a glazing effect has sufficiently good operational properties and can be used for the construction of environmentally friendly buildings and structures.

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SAŽETAK

Efekat glaziranja pri proizvodnji ekološki prihvatljivih keramičkih fasadnih obloga

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U ovom radu su predstavljeni rezultati uporednih studija na temu ekološki prihvatljivih keramičkih materijala na bazi nisko-plastičnih glina sa dodatkom tri različita odnosa galvanskog mulja, borne kiseline i titan-dioksida. Eksperimentalni uzorci su dobijeni presovanjem pri pritisku od 15 MPa i pri maksimalnoj temperaturi pečenja od 1050 °C. Pre toksikoloških analiza dobijeni su ekstrakti ispitivanih materijala u neutralnom i kiselom medijumu na dnevnom nivou. Toksikološka bezbednost materijala procenjena je primenom metode za određivanje mortaliteta po Dafniju i upoređivanjem maksimalno dozvoljenih koncentracija teških metala za pijaću vodu i vodu za domaćinstvo sa koncentracijama teških metala udobijenim ekstraktima. Dobijeni rezultati pokazuju da kombinovana primena korišćenih aditiva rezultira efektom glaziranja površine keramičkih čestica, tako da je opisanim postupkom moguće proizvesti ekološki prihvatljiv materijal koji se odlikuje visoko-kvalitetnim svojstvima. Upotreba nisko-plastične gline i galvanskog mulja proširuje sirovinsku bazu za proizvodnju keramike i omogućava odlaganje ekološki opasnih jedinjenja koja sadrže teške metale koji se nalaze u galvanskom mulju. Keramički materijali na bazi prikazanog sirovinskog sastava mogu se koristiti za proizvodnju obloga za spoljne zidove zgrada i građevina generalno.

Ključne reči: bezbednost životne sredine; vitrifikacija; glina niske plastičnosti; mulj za galvanizaciju; teški metali

Validation of a method for ethanol analysis in biological and non-biological samples and its toxicological application

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Abstract

A simple, cost-effective and fast headspace gas chromatography method coupled with flame ionization detection (HS-GC/FID) for determination of ethanol was developed and validated for clinical and forensic toxicology purposes. HS-GC/FID is often used for alcohol determination in different biological and non-biological samples. The calibration dependence of the method was linear in the range from 0.15 to 4.00 g dm^{-3} (r^2=0.999) with adequate accuracy (99–106 %) and precision. The limit of detection (LOD) was 0.006 g dm^{-3}. The method was quantitative (LOQ) above 0.020 g dm^{-3}. The new method was successfully used for determination of ethanol in biological samples of intoxicated patients, car accidents participants, participants in criminal acts, and postmortem samples, non-biological samples such as alcoholic beverages, alcohol-based herbal preparations, cosmetic preparations, *etc.* This method is easy to perform, making it suitable not only for the routine applications in clinical biochemistry and forensic laboratories, but also in different fields of industry (*e.g.* for pharmaceutical preparations, cosmetics, dietary supplements, *etc.*). Some of the applications for ethanol determination in different samples related to various clinical-forensic cases are presented.

Keywords: headspace; gas chromatography; alcoholic beverages; cosmetics.

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1. INTRODUCTION

Ethanol is a substance of high toxicological significance. Undoubtedly, it is one of the most commonly found psychoactive substances in forensic and clinical toxicology. Evaluation of ethanol in biological samples is requested for legal purposes such as postmortem alcohol evaluation and driving under the alcohol influence, but also in scientific studies of alcohol metabolism. The most common methods are breath analysis in the field and blood analysis in the laboratory [1]. Also, ethanol analysis is required in quality control of alcohol-based herbal preparations, cosmetic preparations, alcoholic beverages, *etc.* Numerous chemical and enzymatic methods have been described to determine ethanol in biological materials [2-5]. Methods for sample preparation are static and dynamic headspace [6-8], and solid-phase microextraction [3,9], while chemical analytical methods are gas chromatography (GC) [6,7,10,11], infrared spectroscopy (IR) [12] and high-performance liquid chromatography (HPLC) [13,14].

High precision and low limits of detection of methods for ethanol determination are a demand for toxicologists because regulations about the upper limit for permitted blood alcohol concentration are becoming stricter during the time. In Serbia, the allowed blood concentration is 0.20 g dm^{-3}. That is the reason for optimization and validation of new methods for ethanol analysis.

Due to the number of samples received, a toxicological laboratory requires a rapid and accurate analytical method for determining ethanol concentration [15]. Headspace gas chromatography with flame-ionization detection (HS-GC–FID) has become a gold standard for ethanol analysis because of ease of automation, accuracy, sensitivity, and specificity [16]. It allows a relatively large number of samples to be analyzed quickly, with a minimal amount of manual

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handling. Due to the complex matrix of biological samples, static headspace sampling is the sampling method used for ethanol analysis because the column and injector are protected so that contamination will not occur; while GC/FID is employed for analyte separation and detection [11,17,18].

This paper aims to validate a quantitative HS-GC/FID method for ethanol assessment in biological and non-biological samples and to present its application in resolving various clinical-forensic cases.

2. EXPERIMENTAL

2. 1. Reagents

Ethanol standards in distilled water (0.15. 0.3. 0.5. 0.8, 1.0. 1.5. 2.0. 3.0. and 4.0 g dm^{-3}) and ethanol standards in whole blood (0.80 and 1.00 g dm^{-3}) were obtained from Medichem Diagnostica GmbH&Co, Germany, while ethanol standards in whole blood (0.30. 0.50 and 1.1 g dm^{-3}) were purchased from ACQ Science GmbH & Co, Germany.

Standard *n*-propanol, which was used as an internal standard (IS), was purchased from Merck, Germany. IS concentration of 0.5 g dm^{-3} was prepared by diluting *n*-propanol with deionized water prepared in-house.

Standards in whole blood were stored at 5 °C, while standards in water were stored at room temperature.

Completely anonymous whole blood samples were used for the validation procedure, which were obtained from ACQ Science GmbH & Co, Germany.

2. 2. Instrumental analysis

The analysis was performed on the GC-2010 Plus (Shimadzu, Japan) gas chromatograph with a flame ionization detector. The system was equipped with an HS-20 Headspace Sampler (Shimadzu, Japan) with 90-sample tray. Zebron BAC1 column (30 m × 0.53 mm × 3.00 µm) was used.

The Headspace sampling system (HS-20) parameters were configured as follows:

- oven temperature = 85 °C,
- sample line temperature = 150 °C,
- transfer line temperature = 150 °C,
- equilibration time = 15 min,
- the time for one cycle = 5 min,
- the sample loop = 1 $cm^{3.}$
- injection time = 0.3 min.

The main advantage of HS-20 sampling system is an overlapping analysis of multiple samples. At the same time, multiple samples are in the equilibration stage, which shortens the overall time for analysis.

The parameters used for GC-2010 Plus are configured as follows:

- hold at 45 °C isothermal for 2.40 min;
- nitrogen (purity 99.9992 %) is employed as a carrier gas, at a constant flow of 30 cm^3 min^{-1};
- the detector gas is a mixture of hydrogen and the air; the flow of hydrogen is 40 cm^3 min^{-1} and the air is 400 cm^3 min^{-1}.
- FID temperature is set to 260 °C.

The analysis of the results and data integration was performed by using the LabSolutions program (Shimadzu, country), while statistical analysis was performed by using Microsoft Excel (Microsoft Corp., USA).

2. 3. Sample preparation

Before starting, all calibration solutions and controls were allowed to equilibrate at room temperature. Whole blood samples were mixed before pipetting.

Standard solutions of ethanol (300 µL) were placed into clean glass headspace vials containing 200 µL of 0.5 g dm^{-3} *n*-propanol as an internal standard (IS). Each vial was sealed with a rubber cap and aluminium crimp seal immediately after addition of the standard.

Depending on the expected concentration, some preparations must be diluted (10, 100, 1000 times) before the analysis.

3. RESULTS AND DISCUSSION

The method for determination of ethanol was validated according to the guidelines established by the International Conference on Harmonization (ICH) and the Scientific Working Group for Forensic Toxicology (SWGTOX) [19].

The selectivity, calibration model (linear), accuracy, precision (interday and intraday), limits of detection and quantification (LOD and LOQ, respectively), carry over are presented.

3. 1. Evaluation of the analytical signal

To improve precision and accuracy, the IS was used so that ethanol and IS peak areas were measured and calculations were carried out considering peak ratios of the analyte to IS.

3. 2. Selectivity

The method demonstrated excellent chromatographic selectivity at the retention times of ethanol and the IS (1.410 and 2.144 min, respectively), which is represented at chromatogram in Figure 1. A selectivity study was conducted analyzing whole blood spiked with possible interferences (methanol, acetone). Interferences with these substances were not observed.

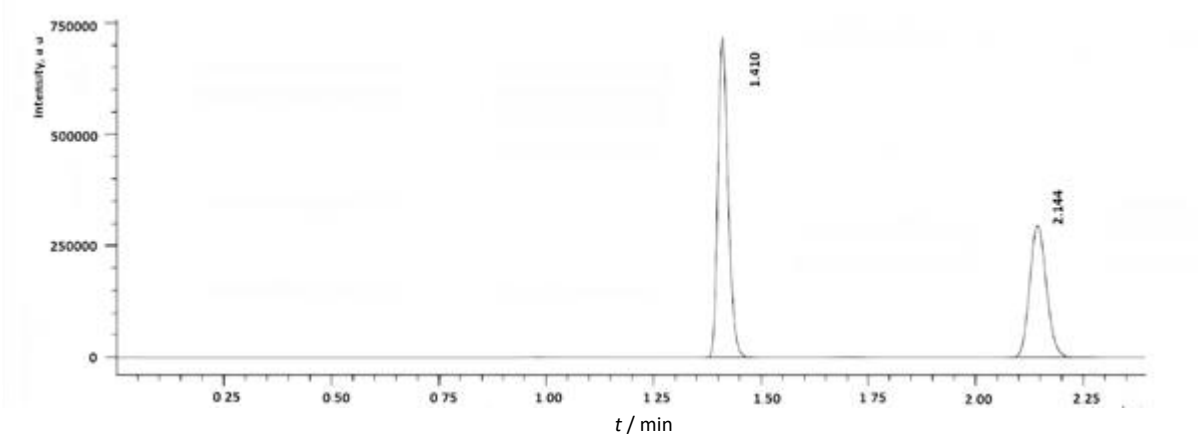

Fig. 1. The representative chromatogram of ethanol (C = 1 g dm^{-3}; R_t = 1.410 min) and n-propanol (C = 0.5 g dm^{-3}; R_t = 2.144 min) standards in whole blood obtained by using Shimadzu HS-GC/FID (HS-20. GC-2010Plus)

3. 3. Matrix-effect

When aqueous ethanol solutions are used for instrument calibration involving a HS-GC technique to quantify this substance, it is necessary to eliminate the matrix effects [20]. The matrix-effect assay was determined using three different whole blood controls, one blank, another containing ethanol, and the last containing both ethanol and IS. Endogenous interference and matrix effect were not observed as peaks were not detected at retention times of analyte and IS in blank whole blood samples. Also, there were no co-eluted peaks with the analyte and IS. All samples were tested in triplicate, in two consecutive days.

Tiscione and coworkers have demonstrated that matrix effects were not occurring between water and whole blood or water and urine standards. They have observed a good correlation for both blood and urine, with r^2 values 0.9999 and 1.0000 respectively, as compared to aqueous standards [21]. Also, selectivity and specificity for all tested compounds in blood, urine and vitreous humor samples was proved in literature [20].

According to the results obtained in the matrix-effect test (matrix effect in whole blood was not observed), as well as results of other authors, it was concluded that aqueous ethanol standards may be used as calibrators and controls when analyzing whole blood, urine and vitreous humour samples [21].

3. 4. Carryover

The sample carryover evaluation was performed by analyzing the ethanol-free whole blood control immediately after the analysis of the standard calibrator with the highest concentration of ethanol in water (4 g dm^{-3}). Carryover was not noticed as peaks were not detected at retention times of the analyte.

3. 5. Linearity

After establishing the chromatographic conditions, the calibration curve was prepared at ethanol concentrations in the range of 0.15–4.00 g dm^{-3}. The calibration model was determined from nine-point calibration curves with calibrators prepared in triplicate as ethanol standards in water. A linear regression of the ratio of the peak area counts of the analyte and IS ($f(C)$) versus the analyte concentration (C) was used to construct the calibration curve. The linear regression equation was:

$$f(C) = 1.94627\ C - 0.0572076 \quad (1)$$

Linearity was obtained with the correlation coefficients r^2=0.9999 and r=0.9999, which proved good linearity (coefficient values above 0.999). The calibration curve is shown in Figure 2.

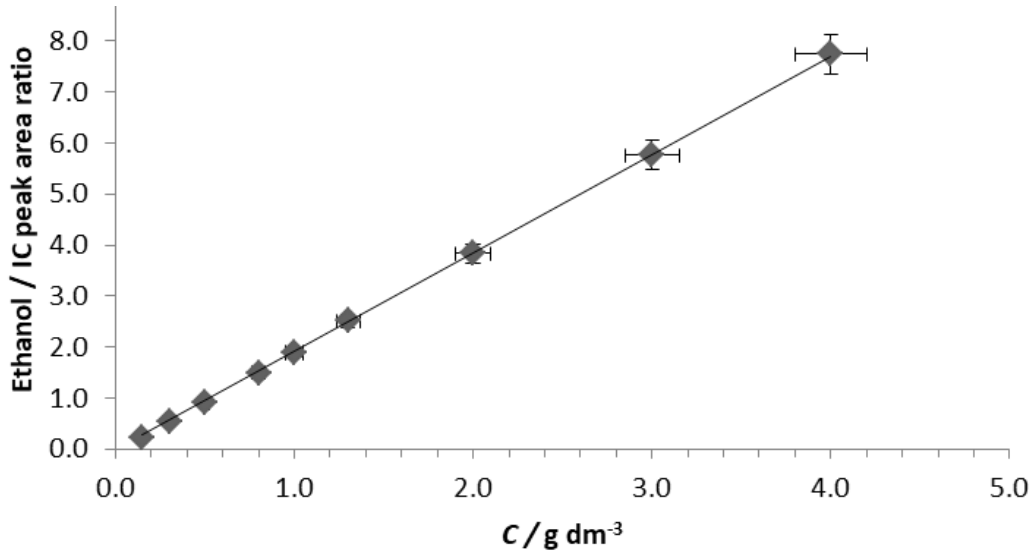

Figure 2. Experimental data of the ratio of peak areas of ethanol and IS (f(C)) vs. ethanol concentration (C) and the best linear fit as the calibration curve for ethanol determination (data present average of n = 3)

Routine laboratory practice requires fast and reliable results. Unknown concentrations of alcohol are present in different samples, so the calibration curve has to be prepared in a wide range of concentrations. The nine-point calibration curve provides the possibility to determine ethanol concentration in all samples, with required precision and accuracy.

3.6. Accuracy

The accuracy of the method was determined by the analysis of whole blood samples. The recovery tests of this analytical procedure were performed by analyzing 5 standard concentrations of ethanol in whole blood in the range 0.30 – 1.10 g dm^{-3}. A recovery rate, bias, standard deviation, and coefficient of variation were obtained, and the results are shown as percentages in Table 1. Accuracy/bias of the assay did not exceed ±10 % over the dynamic range of the assay for the analyte, following SWGTOX guidelines that establish the maximum acceptable bias for ethanol analysis at ±10 % at each concentration level. Coefficients of variation were in the range from 0.85 % calculated for concentration 0.50 g dm^{-3} to 1.59 % for concentration 0.30 g $dm^{-3.}$ while bias was from 0.018 % calculated for concentration 0.50 g dm^{-3} to 5.76 % for concentration 0.30 g dm^{-3}.

Table 1. Recovery test for ethanol in whole blood; bias, standard deviation and coefficient of variation (CV)

Theoretical concentration, g dm^{-3}	Bias, %	Mean measured concentration, g dm^{-3}	Mean recovery, %	SD, g dm^{-3}	CV, %
0.30	5.76	0.3173	105.77	0.0050	1.59
0.50	0.02	0.4991	99.82	0.0042	0.85
0.80	0.93	0.8074	100.93	0.0143	1.77
1.00	0.88	0.9912	99.12	0.0096	0.97
1.10	1.52	1.1167	101.51	0.0112	1.00

3. 7. Precision

Intra-day precision - repeatability, defined as the coefficient of variation, was determined by ten individual replicates of ethanol standard concentration of 1 g dm^{-3}.

The average measured value was 1.0320 ± 0.0059 g dm^{-3}. Values of the obtained standard deviation (0.0059 g dm^{-3}) and coefficient of variation (0.5672 %) indicate good precision for our method, according to ICH [22]. In Table 2 the determined ethanol concentrations and recovery values are presented.

Table 2. Results of ten replicate measurements of the ethanol standard (1 g dm^{-3}) and recovery values

Test number	Measured concentration, g dm^{-3}	Recovery, %
1	1.0437	104.37
2	1.0285	102.85
3	1.0268	102.68
4	1.0311	103.11
5	1.0328	103.28
6	1.0288	102.88
7	1.0276	102.76
8	1.0428	104.28
9	1.0281	102.81
10	1.0301	103.01

Inter-day precision testing using three different concentrations measured three times was performed in two consecutive days and statistical parameters are calculated (Table 3). Coefficients of variation were in the range from 0.03 to 0.32 %. These parameters indicate excellent inter-day precision of this method.

SD values represent the deviation of the mean concentration measured on the second day from the mean concentration measured on the first day.

Table 3. Results of the test for the inter-day precision: standard deviation and coefficients of variation were calculated for 3 measurements and recovery values for for each concentration measured at the second day compared to mean concentration measured at the first day

Concentration, g dm^{-3}			SD, mg dm^{-3}	CV, %	Recovery, %
Standard	Mean measured, first day	Mean measured, second day			
0.3	0.2999	0.2985	0.969	0.32	99.96
					98.46
					100.20
1.0	0.9968	0.9979	0.777	0.08	100.31
					99.74
					100.27
2.0	1.9972	1.9980	0.565	0.03	100.09
					99.98
					100.04

3. 8. Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard deviation of the results for the lowest concentration (0.15 g dm^{-3}) according to equations:

$$LOD = 3\ SD \quad (2)$$

$$LOQ = 10\ SD \quad (3)$$

leading to:

$$LOQ = 3.3\ LOD \quad (4)$$

Based on the results of 6 repeated measurements of the standard with the theoretical concentration of 0.15 g $dm^{-3.}$ which yielded the mean value of 0.152 ± 0.002 g dm^{-3} with CV = 1.122 %, the LOD and LOQ values were calculated as 0.006 and 0.020 g $dm^{-3.}$ respectively. The obtained values are suitable for routine analyses.

3. 9. Comparison of the obtained results with the results in literature

Table 4 presents results of different parameters in validation of methods for ethanol determination published by different authors. The obtained coefficient of determination in our study was high *i.e*. 0.999. Our study has shown a narrow range of recovery values, close to 100 %. Only one method exhibited lower limits of detection and quantification [25] than those determined in the present study.

Table 4. Validation parameters obtained in the present study and studies published in literature

Reference	Linear Range	R^2	Recovery, %	LOD, g dm^{-3}	LOQ, g dm^{-3}
This study	0.15-4.00	0.999	99.12-105.77	0.006	0.02
[23]	0.30-3.5	0.993	91.00 – 109.10	0.099	0.13
[20]	0.1-10	0.990	/	0.005	0.01
[24]	0.5-5	0.9992-0.9999	/	0.050	0.05
[25]	0.075-2.4	0.999	89.0–114.4	0.00053	0.002

3. 10. Application of the validated method

This analytical method validated at the toxicological laboratory of the Institute of Forensic Medicine in Nis was accredited by the Accreditation Body of Serbia, according to ISO/IEC 17025/2005 in 2019 and reaccredited according to ISO/IEC 17025/2017 in 2020. This analytical method is precisely set and described in a standard operative procedure (SOP), which is followed by the laboratory personnel. The SOP is available and controlled by experts in charge of the inspection of the laboratory, in the accreditation process. The SOP contains analytical procedures and validation parameters, including information about participation in external proficiency testing. Proficiency testing (PT) is performed four times a year since 2015. and the toxicological laboratory has passed PT schemes every time.

This validated method has found a wide spectrum of applications in resolving many different forensic cases at the Institute of Forensic Medicine in Nis. Daily, it is used for alcohol determination in biological samples:

- antemortem clinical cases (blood, urine of intoxicated patients)
- antemortem legal cases (blood, urine of participants of a road accident, criminal acts, *etc.*)
- postmortem cases (blood, urine, vitreous humour).

Along with biological samples, ethanol content is also routinely analyzed in non-biological samples (alcohol beverages). Also, the method provides the possibility for quantification of potentially present methanol.

Here we present some chosen obtained results of analyses of different samples.

3. 10. 1. Herbal preparations

There have been few cases of people which blood tests for alcohol content showed the presence of alcohol although they claimed that they had not consumed any alcohol beverages, but only herbal preparations containing alcohol. The reason is that ethanol is used as an excipient in various pharmaceutical formulations. Ethanol contents in pharmaceutical products vary in different formulations; higher ethanol concentrations are most commonly used in liquid formulations such as syrups, solutions and suspensions [26]. In one syrup, 1.6 wt.% (16 g dm^{-3}) of ethanol was determined, while even 49 wt.% of ethanol was found in another herbal tincture.

3. 10. 2. Non-alcoholic beverages

There were several cases of symptoms of alcohol intoxication, but the persons in question did not consume any alcoholic beverage on purpose. The suspected drinks were sent for the toxicological analysis. In some cases, the analysis has shown the presence of ethanol, while in the others, along with ethanol there were other psychoactive substances. For example, a liquid resembling water has shown the presence of 1.08 g dm^{-3} of ethanol along with the anxiolytic and sedative drug bromazepam.

3. 10. 3. Cosmetic preparations

It is known that cosmetic preparations contain denatured ethanol (predominantly ethanol with less than 5 % methanol), to prevent abuse by alcoholics. Sometimes, intoxications with cosmetic preparations happen, accidentally or on purpose. In one case, a cosmetic preparation was analyzed for ethanol and methanol contents, showing only methanol presence. The chemical composition of this cosmetic preparation was not in accordance with listed chemical compounds. Also, the concentration of methanol in preparation was above the permitted concentration (35.14 wt.%). According to the methanol toxicological profile, it is extremely life-threatening to consume such cosmetic preparations. In this case it is more dangerous, because the concentration of methanol was above the permitted value.

4. CONCLUSION

A rapid, highly sensitive and reliable headspace-GC–FID method was established for ethanol measurement and was validated in terms of linearity, selectivity, accuracy, precision, and detection and quantification limits. It was verified that this method for ethanol determination is applicable in routine diagnostics and monitoring for forensic–toxicological and analytical purposes.

The results indicated good linearity ranging between ethanol concentrations of 0.15 and 4.00 g dm^{-3} at sufficient accuracy and precision. Since samples can be analyzed directly, without special preparation, results can be obtained rapidly (2.40 min run time).

This method is easy to perform, making it suitable not only for the routine application in clinical biochemistry and forensic laboratories, but also in different fields of industry (pharmaceutical preparations, cosmetics, dietary supplements, *etc.*). We have shown that it can be applied to numerous samples, biological and non-biological. Determination of ethanol content is of high importance for resolving important forensic cases, as presented in this paper.

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SAŽETAK

Validacija metode za analizu etanola u biološkim i nebiološkim uzorcima i njena primena u toksikologiji

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(Stručni rad)

Jednostavna, ekonomična i brza metoda „head-space“ gasne hromatografije sa plamenojonizujućim detektorom (*engl.* headspace gas chromatography coupled with flame ionization detection, HS-GC/FID) za određivanje etanola, razvijena je i validirana radi primene u kliničke i forenzičke toksikološke svrhe.HS-GC/FID se često koristi za određivanje etanola u različitim biološkim i nebiološkim uzorcima. Kalibraciona kriva metode je bila linearna u rasponu od 0,15 do 4,00 g dm^{-3} (r^2 = 0,9999) sa adekvatnom tačnošću (99,12–105,77 %) i preciznošću. Granica detekcije (*engl.* limit of detection, LOD) bila je 0,006 g dm^{-3}. Metoda je bila kvantitativna (*engl.* limit of quantification, LOQ) iznad koncentracije etanola od 0,02 g dm^{-3}. Nova metoda je uspešno korišćena za određivanje etanola u biološkim uzorcima pacijenata, učesnika saobraćajnih nezgoda, izvršioca krivičnih dela, postmortem uzorcima, nebiološkim uzorcima poput alkoholnih pića, biljnih preparata na bazi alkohola, kozmetičkih preparata, itd. Metoda je jednostavna za izvođenje, što je čini pogodnom ne samo za svakodnevnu praksu kliničko-biohemijskim i forenzičkim laboratorijama, već i u različitim poljima industrije (farmaceutski preparati, dijetetski suplementi, kozmetički preparati...). Takođe, prikazana je i primena ove metode za određivanje sadržaja etanola u različitim uzorcima povezanih sa kliničko-forenzičkim slučajevima.

Ključne reči: headspace, gasna hromatografija, alkoholna pića, kozmetika

Overview of the round table "Medicine and engineering: An inexhaustible source of challenges for cooperation between medical doctors and engineers"

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Abstract

The Academy of Medical Sciences of the Serbian Medical Society (AMS-SMS) and the Academy of Engineering Sciences of Serbia (AESS) have jointly organized the first scientific and expert round table entitled Medicine and Engineering: An Inexhaustible Source of Challenges for the Cooperation of Doctors and Engineers. Biomedical engineering (BME) is a young scientific discipline, in which medical doctors and engineers have united their knowledge with the aim to solve new substantial challenges in the modern society including but not limited to the areas of preventive, regenerative and reconstructive medicine. Here, a short overview of this round table is presented, which was organized for the first time in Serbia.

Keywords: biomedical engineering; scientific and expert meeting; Academy of Medical Sciences of the Serbian Medical Society; Academy of Engineering Sciences of Serbia.

Available on-line at the Journal web address: http://www.ache.org.rs/HI/

BOOK AND EVENT REVIEW

UDC: 005.745-021.372:(61+62)

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INTRODUCTION

The Academy of Medical Sciences of the Serbian Medical Society (AMS-SMS) and the Academy of Engineering Sciences of Serbia (AESS) have signed a Protocol on cooperation on February 26, 2020, with the aim to contribute to the exchange of knowledge and experience of medical doctors and engineers and to provoke new multidisciplinary research studies. Considerable advancements in medicine have been achieved in the last 2 decades of the 21st century by collaborative efforts of medical and engineering experts in various multidisciplinary research teams worldwide. With the aim to solve more efficiently many old, but also new challenges in preventive, reconstructive, and regenerative medicine as well as in broader fields, medical doctors and engineers have joined their work in the frame of a new scientific discipline – biomedical engineering (BME). Such multidisciplinary teams have achieved significant advancements in almost all fields of BME: bioinformatics, biomechanics, biomaterials science, tissue engineering, pharmaceutical engineering, medical equipment, and devices as well as in clinical and rehabilitation engineering [1].

The first scientific meeting organized jointly by AMS-SMS and AESS entitled *Medicine and Engineering: An Inexhaustible Source of Challenges for the Cooperation of Doctors and Engineers* was held in the Ceremonial Hall of the Serbian Medical Society on June 24, 2021, while respecting all preventive measures against the COVID-19 pandemic. After the welcoming words of the Presidents of the academies, Ljibica Đukanović (AMS-SMS) and Branko Kovačević (AESS), the members of AESS and AMS-SMS presented their most significant results achieved in the field of BME:

- Nenad Ignjatović, A bridge over great challenges in medicine: connecting doctors and engineers (AESS);
- Miroljub Adžić, Lesser known connections between mechanical engineering and medicine (AESS);
- Vladimir Nešić, Application of automation in medicine (Institute Mihajlo Pupin);
- Dragan Dankuc, Artificial inner ear (AMS-SMS);
- Bojana Obradović, Biomimic bioreactor systems for tissue and tumor engineering (AESS);
- Dragoslav Stamenković, Biomedical engineering in dentistry (AMS-SMS).

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Paper accepted: 30 June 2021

Figure 1. Opening of the meeting: Ljubica Đukanović (President of AMS-SMS) and Branko Kovačević (President of AESS)
Slika 1. Otvaranje skupa: Ljubica Đukanović (predsednica AMN-SLD) i Branko Kovačević (predsednik AINS).

After the presentations, discussion followed with the exchange of opinions between medical doctors and engineers in solving current challenges in different fields of medicine and engineering. The presented talks portraying successful collaboration of medical doctors and engineers were appraised with the accent on the multilayerdness and complexity of the shown research studies. One of the conclusions of the meeting is that the creation of collaborative research teams and multidisciplinarity comprising different BME fields form a certain way to excellence and progress. It is expected that the meeting will lead to new collaborative projects of engineers and medical doctors in Serbia, which will also contribute to promotion of both Academia. It was also concluded that educational degrees in BME, which have been introduced at different universities in Serbia over recent years, should be included in the job catalogue in the public sector.

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Osvrt na okrugli sto "Medicina i inženjerstvo: neiscrpni izvor izazova za saradnju lekara i inženjera"

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Izvod

U organizaciji Akademije medicinskih nauka Srpskog lekarskog Društva (AMN-SLD) i Akademije inženjerskih nauka Srbije (AINS) organizovan je po prvi put naučno-stručni okrugli sto pod nazivom Medicina i inženjerstvo: neiscrpni izvor izazova za saradnju lekara i inženjera. Biomedicinsko inženjerstvo (BI) je mlada naučna disciplina u kojoj su inženjeri i lekari objedinili svoja znanja i iskustva, a u cilju rešavanja novih velikih izazova koje savremeno društvo nameće u oblasti preventivne, regenerativne i rekonstruktivne medicine i šire. U kratkom izvestaju je prikazan Okrugli sto na ovu temu koji je po prvi put organizovan u Srbiji.

Keywords: biomedicinsko inženjerstvo; naučno-stručni skup; Akademija medicinskih nauka Srpskog lekarskog Društva; Akademija inženjerskih nauka Srbije.

PRIKAZ KNJIGA I DOGAĐAJA

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UVOD

Akademija medicinskih nauka Srpskog lekarskog društva (AMN-SLD) i Akademija inženjerskih nauka Srbije (AINS) su 26. februara 2020. godine potpisale protokol o saradnji sa ciljem da se doprinese razmeni iskustava i znanja inženjera i lekara i podstaknu nova multidisciplinarna istraživanja. U poslednje dve dekade 21. veka veliki napredak u medicini ostvaren je zajedničkom saradnjom lekara i inženjera u različitim multidisciplinarnim istraživačkim timovima širom sveta. U cilju efikasnijeg, bržeg i uspešnijeg rešavanja mnogih starih ali i novih izazova u preventivnoj, rekonstruktivnoj i regenerativnoj medicini ali i šire, inženjeri i lekari su se povezali pod okriljem nove naučne discipline – biomedicinsko inženjerstvo (BI). Multidisciplinarni istraživački timovi sačinjeni od inženjera i lekara ostvarili su značajne napretke u gotovo svim oblastima BI: bioinformatici, biomehanici, biomaterijalima, inženjerstvu tkiva, farmaceutskom inženjerstvu, medicinskoj opremi i uređajima, kao i u kliničkom i rehabilitacionom inženjerstvu [1].

Prvi naučni sastanak u zajedničkoj organizaciji AMN-SLD i AINS održan je 24. juna 2021. godine, u svečanoj sali Srpskog lekarskog društva u Beogradu sa temom Medicina i inženjerstvo: neiscrpni izvor izazova za saradnju lekara i inženjera, a uz poštovanje svih mera protiv pandemije COVID-19. Nakon pozdravnih reči predsednika akademija, Ljibice Đukanović (AMN-SLD) i Branka Kovačevića (AINS) članovi AINS i AMN-SLD su predstavili svoje najznačajnije rezultate ostvarene u oblasti BI:

- Nenad Ignjatović, Most iznad velikih izazova u medicini: povezivanje lekara i inženjera (AINS);
- Miroljub Adžić, Manje poznate veze mašinstva i medicine (AINS);
- Vladimir Nešić, Primena automatike u medicini (Institut Mihajlo Pupin);
- Dragan Dankuc, Veštačko unutrašnje uvo (AMN-SLD);
- Bojana Obradović, Biomimični bioreaktorski sistemi za inženjerstvo tkiva i tumora (AINS);
- Dragoslav Stamenković, Biomedicinsko inženjerstvo u stomatologiji (AMN-SLD).

Nakon održanih predavanja usledila je diskusija, međusobna razmena mišljenja lekara i inženjera u rešavanju aktuelnih izazova u različitim oblastima medicine i inženjerstva. Diskutovano je o održanim predavanjima u kojima je prikazana uspešna saradnja inženjera i lekara, kao i o slojevitosti i kompleksnosti prikazanih istraživanja. Formiranje zajedničkih istraživačkih timova i multidisciplinarnost u različitim oblastima BI siguran je put ka izvrsnosti i napretku, jedan je od zaključaka ovog skupa. Očekivano je da bi održani sastanak vodio ka novim saradnjama inženjera i lekara u našoj zemlji, što bi takođe doprinelo promocijama obe akademije. Obrazovni profil Biomedicinsko inženjerstvo koji je

poslednjih godina uveden na različitim univerzitetima u Srbiji potrebno je uneti u katalog radnih mesta u javnim službama, takođe je jedan od zaključaka sa ovoga skupa.

Slika 2. Auditorijum okruglog stola u svečanoj sali Srpskog lekarskog društva
Figure 2. Audience at the round table in the Ceremonial Hall of the Serbian Medical Society

LITERATURA

[1] Ignjatović N, Mitković M, Obradović B, Stamenković D, Dankuc D, Manić M, Grbović A, Kovačević B, Đukanović Lj. Interdisciplinary crossover for rapid advancements-collaboration between medical and engineering scientists with the focus on Serbia. *Srp Arh Celok Lek.* 2021; 149: 229-235 https://doi.org/10.2298/SARH210110021D

Осврт на VII међународни конгрес „Инжењерство, екологија и материјали у процесној индустрији“

Драган Вујадиновић и Мирјана Берибака

Технолошки факултет Зворник, Универзитет у Источном Сарајеву, Република Српска, Босна и Херцеговина

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Кључне ријечи: EEM2021, хибридни догађај, инжењерство и технологија, хемија.

Available on-line at the Journal web address: http://www.ache.org.rs/HI/

VII међународни конгрес *Инжењерство, екологија и материјали у процесној индустрији* - EEM2021 је одржан од 17. до 19. марта 2021. године у хотелу Термаг на Јахорини (Република Српска, Босна и Херцеговина) у организацији Технолошког факултета Зворник Универзитета у Источном Сарајеву.

Хибридна организација - и лично и на дигиталној платформи
Hybrid organization – both in person and on a digital platform

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Овај научни скуп се одржава сваке двије године, а започео је са радом прије дванаест година. Треба нагласити да је у раду свих скупова до сада учествовало око 1000 научних и стручних радника, са преко 1300 радова из више од четрдесет земаља. EEM2021 је први конгрес у историји наше установе који је организован као хибридни догађај, уживо и на виртуелној платформи, у складу са нормама социјалне дистанце због пандемије ковида 19. Овакав начин организације скупа је омогућио учесницима из цијелог свијета да присуствују пленарним предавањима и предавањима по позиву, као и да учествују у е-постер сесији. Званични језик рада на конгресу је био енглески.

EEM2021 је окупио еминентне истраживаче и професоре из преко 30 земаља. Регистровано је 175 учесника, а укупно је пријављено 228 наслова. Радни дио конгреса одвијао се кроз седам пленарних предавања, седам предавања по позиву, те постер презентације из пет тематских области (Инжењерство и технологија, Екологија, Материјали, Хемија и Остале области).

Програм конгреса је обухватио сљедеће области:

- Хемијско и електрохемијско инжењерство,
- Прехрамбено инжењерство и биотехнологија,
- Еколошко инжењерство,
- Материјали и карактеризација материјала,
- Нанотехнологије,
- Неорганска хемија и технологија
- Органска хемија и технологија, полимери,
- Технологија плазме,
- Енергетска ефикасност и обновљиви извори енергије,
- Текстилно инжењерство,
- Корозија и заштита материјала и термоенергетских постројења,
- Металургија,
- Менаџмент у процесној индустрији,
- Остало.

Отварање скупа EEM2021 / Opening of the EEM2021

У име Технолошког факултета Зворник као организатора, скуп је поздравио др Драган Вујадиновић, предсједник Организационог одбора конгреса, истакавши да је за овогодишњи скуп владало велико интересовање, што се види по броју пријављених наслова и учесницима који долазе из 31 земље. Највеће интересовање је исказано у областима инжењерства, технологије, хемије и животне средине.

С обзиром на тренутну епидемиолошку ситуацију, предсједник Академије наука и умјетности Републике Српске, академик Рајко Кузмановић, није могао да присуствује свечаном отварању, али је послао писмо у којем је похвалио овогодишњу организацију конгреса и истакао да је Технолошки факултет Зворник постао препознатљив по овом међународном скупу не само у Републици Српској и БиХ, него и у региону.

У име спонзора скуп је поздравио и мр Зоран Петковић, генерални директор компаније „Зеохем“ а.д., при чему је истакао да су скупови као што је EEM2021 одлична подршка компанијама попут Зеохема и да научницима и инжењерима пружају најновије информације из области хемијског инжењерства и технологије.

У име Универзитета у Источном Сарајеву, Конгрес је поздравио и свечано отворио др Марко Гутаљ, проректор за међународну сарадњу и осигурање квалитета. Проректор Гутаљ је напоменуо да скуп сваке године добија на значају у различитим међународним организацијама и да је то научни скуп сврстан у прву категорију по класификацији Министарства за научнотехнолошки развој, високо образовање и информационо друштво Републике Српске.

EEM2021 представља добру прилику за размјену идеја, јачање постојећих и стварање нових академских мрежа, као и за подстицање дијалога између академске заједнице, јавних институција, приватног сектора и организација по питању најновијих глобалних и регионалних трендова у процесној индустрији. У овим изазовним временима, очигледна је важност изврсности научних и технолошких истраживања за одрживост процесне индустрије.

Пленарно предавање путем Zoom платформе / Plenary lecture via Zoom platform

Уводно пленарно предавање на скупу је одржао др Џорџ Дедусис (Школа за здравствене науке и образовање, Универзитет Харокопио у Атини, Грчка) на тему „Третман мастихом код гојазних људи са НАФЛД дијагнозом; *MAST4HEALTH* програм“. Након тога, пленарно предавање је одржала др Ивана Смичиклас (Универзитет у Београду, Институт за нуклеарне науке „Винча“, Србија) на тему „Валоризација секундарних извора фосфора: примјенљивост биолошког апатита у ремедијацији и рехабилитацији тла“.

Другог дана су одржана још четири пленарна предавања: др Филип Кокаљ (Машински факултет, Универзитет у Марибору, Словенија) – „Од отпада до енергије у контексту циркуларне економије и управљања отпадом у Европској унији“; др Дејан Поповић (Српска академија наука и умјетности, Србија) – „Гдје инжењерство среће природу: екстерна контрола сензоримоторних система са инвалидитетом“; др Урош Цвелбар (Институт Јожеф Стефан, Словенија) – „Плазма као алат у деконтаминацији природних токсина“ и др Константинос Георгиу (Пољопривредни универзитет у Атини, Грчка) – „Елементална метаболомика“.

Трећег дана одржано је пленарно предавање од стране др Апостолиса Кутинаса (Одјељење за прехрамбену науку и технологију, Пољопривредни универзитет у Атини, Грчка) на тему „Развој биорафинерије за одрживу производњу био-заснованих производа у оквиру циркуларног био-економског контекста“.

Поред пленарних предавања, одржано је и седам предавања по позиву, једна презентација КОСТ пројекта, двије презентације спонзора и 185 е-постера од укупно 214 пријављених наслова, док су уживо изложена 43 постера.

Након завршене постер секције, одржан је округли сто и састанак чланова Научног одбора на коме су чланови комисије заједно са члановима Научног одбора дискутовали о постер секцији, квалитету појединих радова, као и актуелној проблематици у области инжењерства и технологије, екологије и материјала у процесној индустрији. Комисија је, на основу увида у квалитет радова и изглед постера, предложила да се за прве ауторе четири одабрана рада обезбиједи бесплатна котизација за VIII међународни *конгрес Инжењерство, екологија и материјали у процесној индустрији*, EEM2023.

Дио постер презентација које су изложене уживо на Јахорини
Part of the poster presentations that were exhibited live on Jahorina

Покровитељи овог научног скупа су били Академија наука и умјетности Републике Српске и Министарство за научнотехнолошки развој, високо образовање и информационо друштво Републике Српске. Суорганизатори овогодишњег скупа су били Савез инжењера и техничара Србије, Технолошко-металуршки факултет

Универзитета у Београду, Институт за физику Универзитета у Београду, Србија, Прехрамбено-технолошки факултет, Осијек, Хрватска, и Московски државни универзитет за прозводњу хране из Русије. Организација конгреса је подржана од стране три међународне организације – *ISEKI-Food Association*, *European Federation of Chemical Engineering* и *COST Association* – а учесници су имали прилику да рад са конгреса објаве у неком од часописа издавача *Springer Nature*, *Wiley*, Савеза хемијских инжењера Србије и Московског државног универзитета за прозводњу хране из Русије *(SN Applied Sciences, Journal of Food Processing and Preservation, Health, Food & Biotechnology*, *Hemijska industrija* и *Journal of Engineering & Processing Management*).

Скуп је затворен 19. марта 2021. године, при чему је истакнуто да је, упркос пандемији, EEM2021 одговорио постављеним циљевима, што показује велики број учесника из земље и иностранства, као и велики број изложених радова посвећених проблемима и темама које су дефинисане националним стратегијама или међународним пројектима.

ABSTRACT

Overview of the 7th International Congress “Engineering, Environment and Materials in Process Industry”

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(Book and Event Review)

The 7th International Congress “Engineering, Environment and Materials in Process Industry” – EEM2021, was successfully held on the mount Jahorina, from March 17th to March 19th, 2021. The EEM2021 congress gathered eminent researchers and professionals from over 30 countries. A total of 228 papers were submitted, and the fields of greatest interest were chemical engineering and technology, food technology, biotechnology, materials, and environmental sciences. This year’s event was supported by two internationally renowned scientific publishers – Springer-Nature and Wiley, who were kind enough to offer their scientific journals for the research results to be presented, together with other renowned EEM2021 supporting publications – SN Applied Sciences, Journal of Food Processing and Preservation, Health, Food & Biotechnology, Hemijska industrija, and the Journal of Engineering & Processing Management. Due to the COVID-19 outbreak, we were forced to adapt to this new situation, and this was the first time in the history of the Faculty of Technology Zvornik, University of East Sarajevo, that the congress was organized as a hybrid event – both in person and on a digital platform. This mode of organization enabled participants from all over the world to attend plenary lectures and invited speakers’ presentations, as well as to participate in an online poster session. The International Congress “Engineering, Environment and Materials in Process Industry” once again proved to be an opportunity for members of the scholarly community to exchange the results of their work internationally and gain insight into the possibilities of applying their research results in practice, despite the ongoing challenges of the modern world. The Organizing Committee and members of Faculty of Technology Zvornik would like to thank all the researchers, delegates, reviewers, endorsers, co-organizers and sponsors for their contribution to EEM2021. We would also like to invite researchers to join us in our next congress, which we hope will be held under much more fortunate conditions, and members of the Organizing Committee will do their best to outdo themselves and make the following event even better in every manner.

Keywords: EEM2021, hybrid event, engineering and technology, chemistry.