Hydrolysis of sunflower seed meal lignocellulosic fraction by free and immobilized cellulases

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Lignocellulosic biomass is widely abundant in nature and recognized as potential renewable energy source. Its efficient transformation into bio-based fuels is enabled only after adequate pretreatment, followed by enzymatic sacharification and microbial fermentation. Hereby we present application of two cellulase preparations – from *Aspergillus niger* and *Trichoderma reesei* (Celluclast®) in treating sunflower seed meal lignocellulosic fraction (SSMLF). Temperature and pH optimums of two enzymes were determined – 52 °C and pH4.8 for *A. niger* cellulase and 55 °C and pH4.5 for Celluclast®. At optimized conditions, milled SSMLF was hydrolyzed by both biocatalysts. With *A. niger* cellulase higher initial reaction rates were accomplished and yield of 70 mM glucose equivalent was obtained with 6 % (w/v) of enzyme after 6 hours. On the other hand, application of Celluclast® led to lower initial reaction rates and yielded 25 mM of glucose equivalent with 10 % (v/v) of enzyme. To ensure cost-effective application of *A. niger* cellulase, the possibility of its immobilization on different supports was investigated. By using porous methacrylate-based carrier with C6 spacer arm and primary amino groups – Lifetech™ ECR8409, preparation with highest activity was produced. This preparation was successfully applied in saccharification of SSMLF and showed unchanged catalytic efficiency comparing to free enzyme.

INTRODUCTION

Sunflower seed meal is produced in large quantities as a byproduct of oil extraction industry and is currently used as a livestock feed for ruminants and as a fertilizer (Bautista et al., 1990). Within fractionation process, high quality protein concentrate and isolate could be produced from this material. Different approaches could be applied for that purpose, including wet and dry procedures (Kumar & Sharma, 2017). As a waste-product, lignocellulose rich hulls are being obtained, hence ideally suited for further processing. Lignocellulose represents complex matrix composed of three basic biopolymers – cellulose, hemicellulose and lignin – organized into the ununiformed tridimensional structures of various morphologies and structures. Conversion of lignocellulosic substrates, including sunflower seed meal lignocellulosic fraction (SSMLF), into products with high added value such as biofuels and fine chemicals requires multi-step processing comprised of thermo-mechanical, chemical or biological pretreatment, subsequent enzymatic hydrolysis (most commonly by microbial cellulases) and final microbial fermentation.

Cellulases (EC 3.2.1.) are enzymes which catalyze hydrolysis of cellulose and could demonstrate endoglucanase, exoglucanase and *β*-glucosidase activity. Currently, cellulases are widely applied in numerous industry branches such as food, pharmaceutic, textile, paper and pulp industry, as well as agriculture (Bhat & Bhat, 1997; Kuhad et al., 2011). Potential for their cost-effective application in the treatment of lignocellulosic biomass for the production of second generation biofuels is nowadays attracting increasing attention of scientific community (Tran et al., 2019; Sukumaran et al., 2005). Different microbial cellulases were so far used for that purpose. However, relatively high price of available enzyme preparations combined with high dosages required for efficient cellulose hydrolysis represents one of the main challenges for process commercialization.

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Table 1. Characteristics of applied immobilization carriers

Carrier	Functional group	Polarity and porosity
Lifetech™ ECR8305F	amino C2 methacrylate smaller pores	moderately hydrophilic porous
Lifetech™ ECR8309F	amino C2 methacrylate larger pores	moderately hydrophilic porous
Lifetech™ ECR8404F	amino C6 methacrylate smaller pores	moderately hydrophilic porous
Lifetech™ ECR8409F	amino C6 methacrylate larger pores	moderately hydrophilic porous
Lifetech™ ECR1508	stirene/DVB with tertiary amino groups	moderately hydrophobic
Lifetech™ ECR1604	stirene/DVB with quaternary amino groups	moderately hydrophobic
Lifetech™ ECR8806M	octadecyl acrylate	moderately hydrophobic porous
Lifetech™ ECR8285	epoxy/butyl methacrylate	moderately hydrophobic

A convenient method for overcoming these issues is immobilization on adequate solid supports, providing increased operational and storage stability and reusability (Vaz et al., 2016). During the last decades, various microbial cellulases were immobilized onto different nanoparticles (Xu et al., 2011; Gokhale et al., 2013; Lima et al., 2017; Han et al., 2018; Simon et al., 2018), magnetic chitosan microspheres (Miao et al., 2016), kaolin (Lima et al., 2019), etc. However, most of these immobilized preparations are not applicable for the saccharification of natural lignocellulosic substrates due to their high prices.

Main goal of this research was to examine the possibility of application of free and immobilized cellulases for the hydrolysis of SSMLF. Within current study, two commercial cellulase preparations - from *Aspergillus niger* and *Trichoderma reesei* (Celluclast®), were examined as a catalysts for (SSMLF) saccharification. Two biocatalysts were first characterized in terms of pH and temperature optimums and subsequently, at optimized conditions, applied in the SSMLF hydrolysis. Their performances were assessed by analyzing initial reaction rates and final concentration of reducing sugars produced from carboxymethyl cellulose (CMC) as a substrate. Furthermore, eight carriers with different polarity, porosity and functionality (Table 1) were screened as supports for immobilization of *A. niger* cellulase. Lifetech™ series carriers were chosen due to their good mechanical properties, high chemical resistance and facile separation and regeneration after application. Moreover, immobilized biocatalyst with highest cellulolytic activity was tested in a reaction of SSMLF saccharification in order to evaluate its perspective for application in enzymatic pretreatment during bio-fuels production.

MATERIALS AND METHODS

Materials

Two cellulase preparations were used - *Aspergillus niger* cellulase (powder, ≥0.3 units/mg solid, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and *Trichoderma reesei* (Celluclast®, Novozymes, Bagsvaerd, Denmark) as biocatalysts. Substrates were low viscosity carboxymethyl cellulose sodium salt (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and lignocellulosic fraction obtained after fractionation of local sunflower seed meal. Lifetech™ immobilization supports were provided by Purolite Corporation (Bala Cynwyd, PA, USA). For DNS reagent preparation following chemicals were used: 3,5-dinitrosalicylic acid (98 %, Acros Organic, New Jersey, USA), NaOH (p.a., Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and potassium sodium tartarate (p.a., Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Salts used for buffer solutions preparation were: Na_2CO_3 (Zorka Pharma, Šabac, Serbia), sodium citrate (Alkaloid, Skopje, Macedonia) NaH_2PO_4 (Centrohem, Stara Pazova, Serbia), Na_2HPO_4 (Superlab, Belgrade, Serbia), all analytical grade.

Determination of enzyme activity

Activity of free and immobilized cellulases was determined spectrophotometrically by DNS method with 2 % CMC in a buffer (50 mM) as a substrate. When temperature and pH optimums for two preparations were examined, temperature was varied between 35 and 70 °C and pH was in range 3.5-8. Predefined amount of free (1 mg/ml of *A. niger* cellulase and 1 µl/

ml of Celluclast®) or immobilized (25 or 50 mg in 2 ml) cellulase was incubated with substrate at defined conditions (pH, temperature) and samples were taken during time (up to 5 minutes). 50-125 µl of sample were added to 250 µl of DNS reagent to achieve final volume of 0.5 ml and immersed into boiling water bath for 5 minutes. Reaction was stopped by adding 2 ml of distilled water and absorbance was measured at 540 nm. Enzyme activity was determined according to previously described calculations (Ćorović et al., 2017).

Fractionation of sunflower seed meal and hydrolysis of its lignocellulosic fraction

Fractionation of SSM was performed according to standard procedure. By mixing 100 g of minced SSM and 1 l of distilled water, three fractions were obtained – lignocellulosic fraction (LF, upper layer) was separated by perforated spoon from middle layer (liquid fraction) and precipitate (solid fraction). LF was dried at 180 W of microwave power within 10 cycles lasting 5 minutes, until dry matter content was above 98 %.

Hydrolysis reactions were performed at pH 4.8 and 52 °C in a 100 ml Erlenmyer flasks orbitally shaken at 150 rpm in a thermostat. 1 g of substrate was mixed with 10 ml of buffer solution and reaction was initiated by adding predefined amount of free or immobilized enzyme. Samples were taken during reaction and subjected to DNS analysis, according to previously described procedure.

Immobilization procedure

Enzyme immobilization was conducted at room temperature, in 2 ml volume Eppendorf® tubes under stirring on a roller mixer (Stuart, Paris, France). 25 or 50 mg of immobilization support particles were measured and 0.5 or 1 ml of enzyme solution (predefined concentration of enzyme in immobilization buffer) was added. For amino-functionalized immobilization supports, 50 mM phosphate buffer pH 6 was applied in order to enable undisturbed establishment of ionic interactions with enzyme molecules, while for hydrophobic carrier and epoxy-functionalized resin, pH 7 and higher buffer molarity, 1 M, was applied. After 24 h, supernatant was separated, while particles with immobilized enzyme were washed three times with 0.5 or 1 ml of distilled water and used for activity determination. For application in SSMLF hydrolysis the same procedure was applied but with 0.5 g of immobilization support.

RESULTS AND DISCUSSION

Temperature and pH optimum determination

In order to achieve the highest possible performances in terms of catalytic activity, each enzyme should be examined at various reaction temperatures and pH values. For that purpose two cellulases were at first used at temperatures ranging from 35 to 70 °C, while other reaction parameters were kept at constant values and the obtained results are presented in Fig. 1.

As it can be seen, highest hydrolytic activity of both examined biocatalysts was in the mid part of examined range – at 52 °C for *A. niger* cellulase and at 55 °C for Celluclast®. It could be noted that both preparations demonstrated broad temperature optimums. Namely, *A. niger* callulase maintained approximately 50 % of its initial activity at 35 and 70 °C, while Celluclast® showed slightly lower activity at low temperatures (approximately 35 % of initial activity at 35 °C), but significantly higher activity at increased temperatures – over 90 % at 60 °C and over 60 % at 70 °C. When

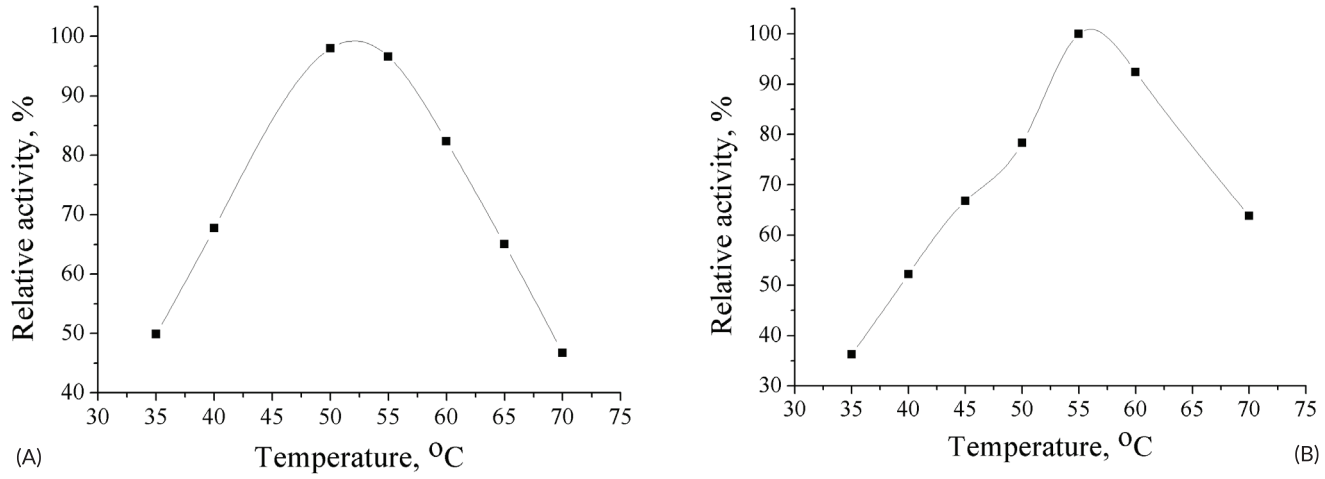

Figure 1. Determination of temperature optimums for cellulases: (A) from A. niger and (B) from T. reesei.

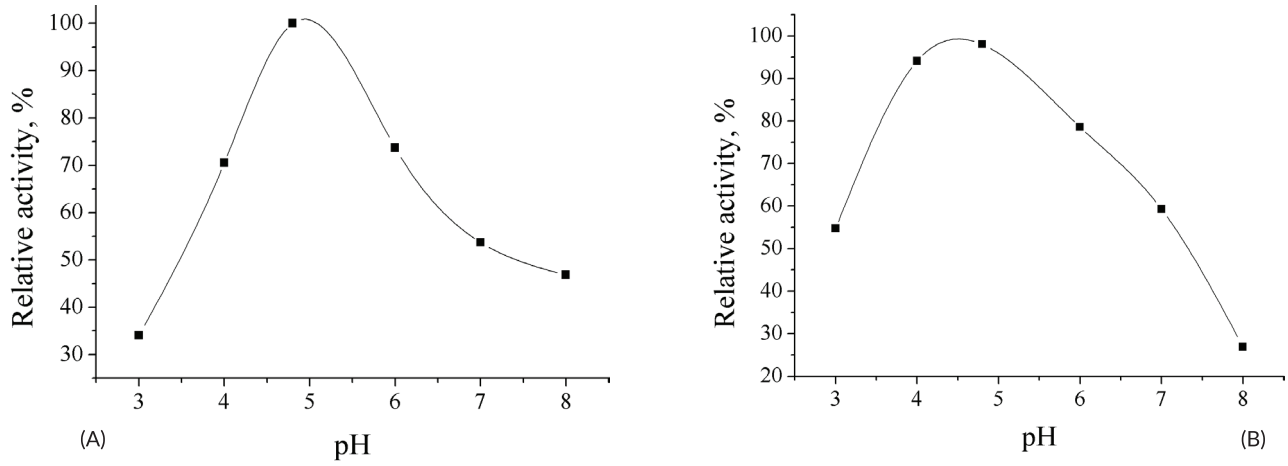

Figure 2. Determination of pH optimums for cellulases: (A) from A. niger and (B) from T. reesei.

it comes to pH optimums (Fig. 2), two preparations demonstrated highest activities at slightly acidic conditions - pH 4.8 for *A. niger* cellulase and pH 4.5 for Celluclast®. *A. niger* cellulase kept over 50 % of its initial activity in a pH range 3.5-6.5, while Celluclast® demonstrated slightly higher activity at different pH values which resulted in over 70 % preserved activity within the same pH range. Determined optimums were applied within the consequent part of the study.

Hydrolysis of sunflower seed meal lignocellulosic fraction

Cellulolitic enzymes are nowadays widely examined in the valorization of agro-industrial waste based on their ability to catalyze saccharification of lignocellulosic substrates and enable their application for second generation bio-fuels production.

Potential of two chosen cellulases for hydrolysis of SSMLF was therefore assessed under previously optimized conditions. The obtained reaction courses are presented in Fig. 3. As it can be seen, higher enzyme loadings led to higher release rates of reducing sugars in both cases and within 6 hours maximum of 70 mM reducing sugars was liberated with *A. niger* cellulase, while with Celluclast only 25 mM of reducing sugars was detected. Also, significantly higher initial reaction rates were accomplished with *A. niger* cellulase. Based on these results, it could be concluded that *A. niger* cellulase possess a much higher potential for SSMLF hydrolysis. Economic feasibility of such application could be notably improved by enzyme immobilization onto suitable solid support since multiple use of the same biocatalyst batch significantly lowers overall process costs. Further steps were, therefore, directed towards examination of different immobilization methods.

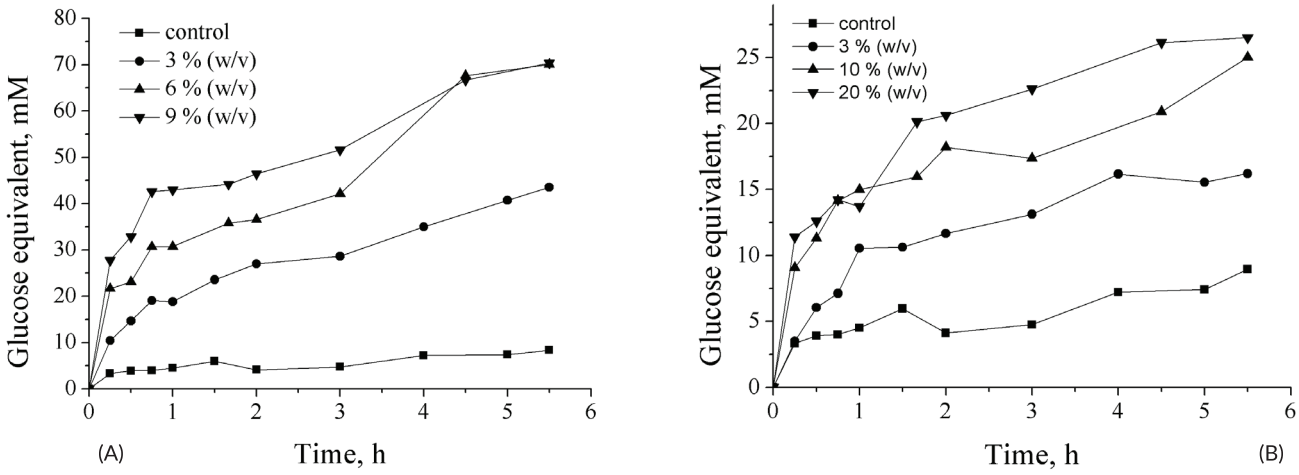

Figure 3. Hydrolysis of SSMLF by different concentrations of free: (A) A. niger and (B) T. reesei cellulase. Reactions were performed at 52 °C, pH 4.8 and 150 rpm, with 1 g of substrate in 10 ml of reaction mixture.

Immobilization of Aspergillus niger cellulase

A. niger cellulase was immobilized onto eight different Lifetech™ carriers with main characteristics shown in Table 2. Displayed performances of the obtained preparations (after 24 h of immobilization) are implying that there is a significant impact of immobilization support functionality, porosity and polarity on their catalytic activity. Regarding carrier hydrophobicity, it is evident that the more hydrophilic support surface was the higher activity of immobilized cellulase was achieved. Namely, highly hydrophobic Lifetech™ ECR8285 octadecyl acrylate based carrier was the least suitable, followed by two moderately hydrophobic supports comprised of styrene/divinyl benzene with tertiary (Lifetech™ ECR1508) and quaternary (Lifetech™ ECR1604) amino groups, and the highest activities were obtained by using four hydrophilic methacrylate carriers with primary amino group. Among them, pronounced influence of pore size (smaller pores 30-60 nm and larger pores 60-120 nm) and functional group spacer arm length (C2 and C6) was observed. It seems that longer spacer arm (C6) provided optimum distance between carrier surface and enzyme molecule, ensuring its unhindered catalytic acting and leading to 37 % higher hydrolytic activity. Furthermore, diffusion of substrate molecules was apparently more efficient through the larger pores, and facilitated approach to the active sites of cellulase molecules immobilized inside the pores enabled approximately 30 % higher activities comparing to preparations obtained by using carriers with smaller pores. Therefore, the most promising support for further examinations is Lifetech™ ECR8409F. Hence, it was applied within the subsequent hydrolysis of SSMLF.

Table 2. Activities of A. niger cellulase immobilized onto diffrent Lifetech™ carriers

Carrier	Activity, IU/g
Lifetech™ ECR8305F	13.47
Lifetech™ ECR8309F	19.17
Lifetech™ ECR8404F	21.30
Lifetech™ ECR8409F	30.24
Lifetech™ ECR1508	17.30
Lifetech™ ECR1604	10.03
Lifetech™ ECR8806M	0
Lifetech™ ECR8285	3.53

Application of immobilized callulase for the hydrolysis of SSMLF

Final goal of current study was to investigate the possibility of the application of the obtained immobilized biocatalyst in the treatment of SSMLF. For that matter, immobilized enzyme was incubated with SSMLF and reaction course was monitored and compared with free enzyme (same amount of CMC units). Initial reaction rates were very similar for two preparations – 30 mM/h, indicating that there were not significant diffusion limitations for substrate to access active sites of immobilized enzyme, probably due to large pore diameter of used carrier. Also, enzyme molecules located inside the pores are protected from the negative influence of shear stress which can cause their deactivation and/or desorption (Sowana et al., 2001). Regarding obtained yields, after 6 h of reaction 68 mM of glucose equivalents were liberated from SSMLF, which is in range with the results obtained using free enzyme. It could be observed that reducing sugar concentration could be even higher if longer reaction times were analyzed. Therefore, immobilized *A. niger* cellulase could be efficiently utilized for the pre-fermentation treatment of SSMLF during bio-ethanol production and all relevant reaction parameters including enzyme and substrate concentration and reaction time should be analyzed in details before potential process scale-up.

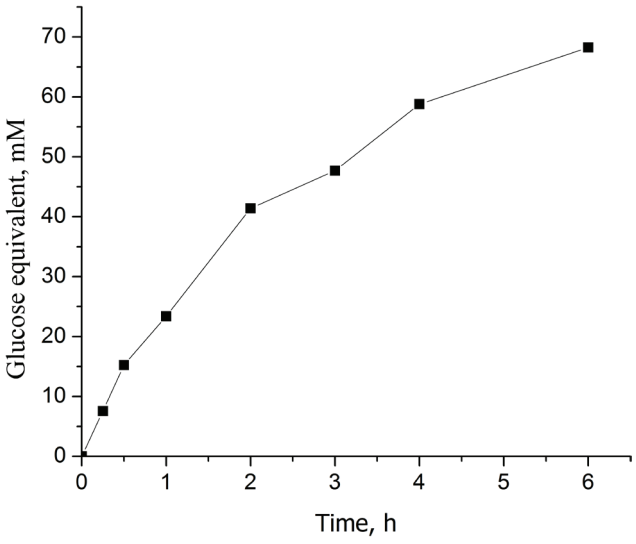

Figure 4. Hydrolysis of SSMLF by immobilized A. niger cellulase. Reactions were performed at 52 °C, pH 4.8 and 150 rpm, with 1 g of substrate in 10 ml of reaction mixture, with 90 IU/g of substrate.

CONCLUSIONS

In the current study, it has been shown that microbial cellulases could be successfully applied for the saccharification of SSMLF as an attractive stock material for bio-ethanol production. The obtained results revealed that *A. niger* cellulase is very promising catalyst for the hydrolysis process, particularly in its immobilized form. By examining a wide range of Lifetech™ immobilization supports with various characteristics (porosity, functionality and polarity), it was proven that hydrophilic, moderately porous carrier with primary amino groups and C6 space arm – Lifetech™ ECR8409F is most suitable for obtaining highly active preparation. Further studies should, therefore, be directed towards more detailed optimization of immobilization conditions and examination of immobilized biocatalyst performances under different operating terms.

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Hidroliza lignocelulozne frakcije suncokretove sačme primenom slobodnih i imobilisanih celulaza

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Ključne reči:
suncokretova sačma, lignoceluloza, celulaza, imobilizacija.

Lignocelulozna biomasa je široko rasprostranjena u prirodi i prepoznata je kao potencijalni obnovljivi izvor energije. Njena efikasna tranfromacija u bio-goriva moguća je samo nakon adekvatnih predtretmana, nakon kojih slede enzimska saharifikacija i mikrobna fermentacija. U okviru ovog rada, prikazana je primena dve različite celulaze producenata *Aspergillus niger* i *Trichoderma reesei* (Celluclast®) u tretiranju lignocelulozne frakcije suncokretove sačme (LFSS). Utvrđeno je da temperaturni i pH optimum iznose 52 °C i pH 4.8 za *A. niger* celulazu i 55 °C and pH 4.5 za Celluclast®. Pod optimizovanim uslovima, mlevena LFSS hidrolizovana je korišćenjem oba biokatalizatora. Primenom *A. niger* celulaze ostvarene su veće početne brzine i nakon 6 sati postignut prinos od 70 mM ekvivalenata glukoze korišćenjem 6 % (w/v) enzima. Sa druge strane, primenom Celluclast®-a postignute su niže početne brzine i dobijeno 25 mM ekvivalenata glukoze sa 10 % (v/v) enzima. U cilju ekonomične primene celulaze producenta *A. niger*, ispitana je mogućnost njene imobilizacije na različite nosače. Korišćenjem poroznog metakrilatnog nosača sa C6 "dugom nožicom" i primarnim amino-grupama - Lifetech™ ECR8409, dobijen je imobilisani enzim najveće aktivnosti. Ovaj preparat uspešno je primenjen u saharifikaciji LFSS, pri čemu je pokazao nepromenjenu katalitičku aktivnot u odnosu na slobodan enzim.