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Title: Immobilization of acid urease enzyme on oak chips for urea elimination from alcoholic beverages

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Keywords: acid urease; alginate; ethyl carbamate; immobilization; oak chips.

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Abstract: In this work the immobilization of acid urease enzyme was carried out to eliminate urea from alcoholic beverages, thus, to prevent the formation of carcinogenic ethyl carbamate. The applied methods were encapsulation in alginate beads and immobilization on oak chips. Alginate can be considered as a well-known example of biocatalyst carriers, therefore, it can be used as a reference point for the comparison. The study on the well-adaptable support material for wine industry that have not been applied so far, called oak chips was the main goal in our experiments. The results showed that the alginate-encapsulated preparations were stable only at pH=5 and neutral media with nearly 800 U g⁻¹ and 400 U g⁻¹ initial activity, respectively. Urea elimination capacities were decreased with an average value of 5.4 U day⁻¹ (pH=5) and 5.0 U day⁻¹ (distilled water). In case of the oak chips-supported urease the initial activities were lower, however, the urea elimination capacity was decreased much slower (0.1 U day⁻¹ after 2nd day) and the optimal immobilization pH was 3 which is advantageous in wine industry.

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Dear Editors,

Attached to this letter, please find the manuscript of an intended “Full length article” entitled “Immobilization of acid urease enzyme on oak chips for urea elimination from alcoholic beverages” for your consideration for publication.

The present work describes for the first time the acid urease enzyme immobilization experiences on oak chips as a well-adaptable support material for wine industry. Since communications on the biocatalyst applications for food industry have been considerably extended in number, the data in this manuscript can be considered quite important for the current researches in this area. Based on our results, it has been demonstrated that the oak chips – the by-product of barrel manufacturing process – could be successfully applied for urease immobilization and the preparation has high storage and operational stability. To our knowledge, the comparative assessment of the oak chips-supported immobilization process with enzyme encapsulation in alginate beads (as a well-known example) for urea elimination is scantily reported and claims novelty to the content of the paper.

Our group has been dealing with various aspects of the enzyme technology and food engineering and a result of this progress, a significant number of papers have been published in highly reputed, quality journals including the *Journal of Food Engineering*, *Food and Bioprocess Technology*, etc. For your reference, a sorted list of recent, relevant publications is as follows:

Bélafi-Bakó, K., Cserjési, P., Beszédes, S., Csanádi, Z., & Hodúr, C. (2012). Berry pectins: microwave-assisted extraction and rheological properties. *Food and Bioprocess Technology*, 5, 1100-1105.

Bélafi-Bakó, K. (2011). Possibilities for removal of glucose from various foodstuffs and food bioprocesses. *New Topics in Food Engineering*, pp. 277-288.

Nemestóthy, N., Gubicza, L., Fehér, E., & Bélafi-Bakó, K. (2008). Biotechnological utilisation of fusel oil, a food industry by-product. *Food Technology and Biotechnology*, 46, 44-50.

Bélafi-Bakó, K. (2008). Simultaneous application of enzymes and membranes in food processing. *Food Engineering Research Trends*, Ed. by Jerrod, M Cantor, Nova Science Publishers, New York, pp. 263-279.

Bélafi-Bakó, K., Eszterle, M., Kiss, K., Nemestóthy, N., & Gubicza, L. (2007). Hydrolysis of pectin by *Aspergillus niger* polygalacturonase in a membrane bioreactor. *Journal of Food Engineering*, 78(2), 438-442.

This work is in our research sequence and we feel that the *Food and Bioproducts Processing* is the most appropriate place for this contribution. The manuscript being submitted now is original, has not been communicated with your journal earlier and is not currently sent for review to any other journal and will not be submitted elsewhere before a decision is made. All the authors agreed with the submission and confirm that all elements of the submission are in compliance with the journal publishing ethics policy.

Thanking you in advance for the time spent in dealing with this manuscript, we are looking forward to hearing from you in the near future.

Sincerely yours,

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Research highlights

- Acid urease was immobilized in alginate beads and on oak chips
- Both preparation method resulted in comparable urea-eliminating activity
- The oak chips-supported enzyme had the most significant storage and operational stability
- Oak chips-supported preparation is better adaptable in wine industry because of its optimal pH

**Immobilization of acid urease enzyme on oak chips for urea elimination
from alcoholic beverages**

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Abstract

In this work the immobilization of acid urease enzyme was carried out to eliminate urea from alcoholic beverages, thus, to prevent the formation of carcinogenic ethyl carbamate. The applied methods were encapsulation in alginate beads and immobilization on oak chips. Alginate can be considered as a well-known example of biocatalyst carriers, therefore, it can be used as a reference point for the comparison. The study on the well-adaptable support material for wine industry that have not been applied so far, called oak chips was the main goal in our experiments. The results showed that the alginate-encapsulated preparations were stable only at pH=5 and neutral media with nearly 800 U g⁻¹ and 400 U g⁻¹ initial activity, respectively. Urea elimination capacities were decreased with an average value of 5.4 U day⁻¹ (pH=5) and 5.0 U day⁻¹ (distilled water). In case of the oak chips-supported urease the initial activities were lower, however, the urea elimination capacity was decreased much slower (0.1 U day⁻¹ after 2nd day) and the optimal immobilization pH was 3 which is advantageous in wine industry.

Keywords: acid urease; alginate; ethyl carbamate; immobilization; oak chips.

1. Introduction

Ethyl carbamate is one of the derivatives of urea and its presence in alcoholic beverages should be avoided. The maximal permitted limit value of ethyl carbamate has been controlled in most of the countries. Recently it has turned out from animal toxicology experiments that the compound is carcinogenic and hazardous probably for humans, as well (Nettleship et al., 1943; Mirvish 1969; Schlatter and Lutz, 1990). Ethyl carbamate may be formed e.g. in preparation of fruit distillates. The amino acid, arginine often occurs in traces in fruits and it is taken as a nutrient by the yeast during alcohol fermentation, then it is converted into urea in the metabolism. Urea is accumulated in the yeast cells up to a certain concentration then it is released to the alcoholic solution where it reacts with ethyl alcohol to form ethyl carbamate. To avoid formation of the compound urea should be eliminated or degraded. For this purpose acid urease enzyme can be used. For real application possibilities the enzyme should be immobilized. In this work the immobilization process was studied and the operation and stability was investigated.

Immobilized enzyme systems have been manufactured and applied for long, their advantages are well-known. These types of solid phase biocatalysts can be prepared by physical and chemical methods (Boross et al., 2008). Acid urease enzyme was immobilized previously on Eupergit and chitosan support, as well, where adsorption-crosslinking and covalent bond methods were used (Andrich et al., 2009, 2010; Bortone et al., 2012; Krajewska et al., 1990; Matsumoto, 1992). To enhance stability and mechanical strength the method was combined with gelatine addition (Li et al., 2011; Sungur et al., 1992). Enzymes can be encapsulated into natural or synthetic cross-linked polymers which cannot be dissolved in water. Enzyme molecules are entrapped in the matrix, while substrates can easily pass through the gel structure to the active centre. Hydrogels are the most popular forms for this type of immobilization and application of Ca-alginate is the most well-known example (DeGroot and Neufeld, 2001; Pannier et al., 2010; Pithawala et al., 2010).

We wanted to select an appropriate support taken into account not only the enzyme and proper immobilization technique point of view but the special aspects of food industry (hygienic, etc.) with particular regard of oenology as the most promising potential applicants. Thus alginate and oak chips were selected. Alginate has been widely used in food industry as

gel forming material, while oak chips belong to the group of approved additives in wine treatment and they have not been applied as support material for enzyme immobilization so far.

Alginate is a popular natural polymer in biocatalysts immobilization technology. In this linear co-polymer β -D-mannuronic acid and α -L-guluronic acid molecules are coupled with 1,4-glycoside bonds. Inside the polymer chain homopolymeric blocks are located from both of monomers, among them mixed fractions occur. Guluronic acid molecules are able to join each other via Ca^{2+} ions, thus guluronic acid content play an important role in the solid state, pore size and stability of the gel (Martinsen et al., 1989). The main advantage of the alginate beads is that they are non-toxic and can be handled easily. By their application the biocatalysts are treated gently, moreover their structure is compatible with most of the enzymes, thus no chemical modification is needed.

Oak barrels have long been used in the wine industry to add desired woody aromas along with butter and vanilla flavours to certain sorts of wines (Sogg, 2002). Nowadays, however oak chips – which are by-product of barrel manufacturing process – are often used instead, since they can add a similar oak character at a tiny fraction of the cost of new barrels (oak chips are referred therefore as “micro-barrels” or “barrel alternatives”). Chips are usually added during the fermentation process so that the oak flavours integrate well with the wine.

In this work the immobilization of acid urease by glutaraldehyde cross-linking on oak chips as a completely new support is presented. The aims of enzyme immobilization in alginate beads by encapsulation were to check if it is really a suitable preparation method as indicated in literature and to create a reference point for efficiency determination of oak chips-supported preparation.

2. Materials and Methods

2.1 Materials

Oak chips (Brouwland, USA) and alginic acid sodium salt (Sigma Aldrich) were used for the immobilization. Urea (Sigma Aldrich), p-dimethyl-amino-benzaldehyde (Sigma Aldrich) and

glutaraldehyde 25% aqueous solution (Reanal, Budapest, Hungary) were applied for model solutions, activity measurements and cross-linking, respectively. All the other compounds (calcium chloride dehydrate, citric acid sodium citrate, ethanol, HCl) were purchased from Reanal (Budapest, Hungary). All the chemicals used were analytically pure.

NAGASPIN (NAGASE ChemteX Corporation, Kyoto, Japan) acid urease enzyme was used in the experiments. NAGASPIN is a powder type acid urease preparation derived from *Lactobacillus fermentum*. Activity of NAGASPIN is 2,300 U g⁻¹ powder, where 1 activity unit is defined as the amount of preparation which catalyses the releasing of 1 μmol ammonia per minute at 37 °C. The optimal pH of NAGASPIN is in the acidic range and it is tolerant to alcohol, and hydrolyses effectively the urea to ammonia and CO₂, thus NAGASPIN is considered effective to eliminate urea from alcoholic beverages.

2.2 Determination of urea

Urea content in the samples was determined by spectrophotometric method in both immobilization techniques (Francis, 2006). The p-dimethyl-amino-benzaldehyde (dissolved in ethyl alcohol) reacts with urea resulting in a colourful product which can be detected easily in 440 nm wavelength. Calibration curve was used for the determination; the volume of the samples was 5-5 ml.

2.3 Encapsulation of acid urease in alginate

60 cm³ 6% aqueous solution was prepared from Na-alginate. Since it is difficult to dissolve alginate, pressure cooker was used which ensured to prepare a homogenous solution. After cooling 60 cm³ aqueous enzyme solution (containing 240 mg urease) was added then the mixture was stirred for 20 minutes at room temperature. The alginate-enzyme solution was added by drop formation into three-fold volume 3% CaCl₂ solution which resulted in gel-bead formation, entrapping the enzyme molecules. To make the gel firm, the beads were held in coagulation bath for 1 hour. Then the beads were washed to chlorine free by distilled water and applied in the experiments.

2.4 Immobilization of urease on oak chips

Various amounts of dried oak chips (with average particle size of 4 mm) were activated in 10 cm³ 2% glutaraldehyde solution (prepared in 0.05 M citrate buffer, pH=5) in shaking incubator (Certomat S) at 120 rpm and 25 °C for 1 hour. The samples were then filtered and washed with distilled water and buffer to remove aldehyde traces. 100 mg acid urease was dissolved in 10 cm³ citrate buffer (pH=5) and the solution with the activated chips were shaken (120 rpm) at room temperature for 2 hours. After immobilization process the samples were filtered and washed to remove the free enzyme.

2.5 Determination of enzyme activity

The initial urea concentration was always 3000 ppm. 10 cm³ partially dried alginate bead (which contained 20 mg immobilized enzyme) was added to 10 cm³ urea solution. The mixture was shaken (100 rpm) at 37 °C in a shaking incubator for 30 minutes. The enzyme activity was determined by measuring the concentration of urea. In case of urease immobilization on oak chips the colourful compound, which was dissolved from oak made it impossible to measure the protein concentration of the solution, thus – in the absence of the quantitative data of the immobilized enzyme amount – the activity could not be calculated by the definition described in chapter 2.1, instead of it another activity definition can be formulated relative to 1 g of carrier. Therefore, the comparison was carried out on the basis of the eliminated urea data.

2.6 Study on the rate-limiting processes

The enzymatic reaction and the substrate diffusion from the bulk phase to the solid biocatalyst surface takes place simultaneously when immobilised preparations are used. To decide which the rate-limiting step is, Yadav's method can be applied (Yadav and Devi, 2002) which is based on the calculation of the reaction and diffusion relaxation time (Eq. 1 and Eq. 2).

$$t_R = C_0 r(C_0)^{-1} \quad (1)$$

$$t_D = D k_{SL}^{-2} \quad (2)$$

where C_0 is the substrate concentration in the liquid phase, $r(C_0)$ is the reaction rate, D is the diffusion constant of the substrate and k_{SL} is the mass transfer coefficient. The process which has a higher relaxation time is slower, thus, it can be considered as a rate-limiting step.

3. Results

3.1 Encapsulation in alginate beads

Activity of the encapsulated urease enzyme was measured at four pH values: pure distilled water was used in one of series and citrate buffers at pH 3, 4 and 5 were used for others. It was found that under lower acidic conditions (pH=3 and PH=4) the beads have swollen, their structure was changed and a pulpy gel was formed, thus the measurements to study the stability were continued only in pH=5 and neutral media (in distilled water).

The activity values of the immobilized urease were determined for a longer period. The results showed that acidic buffer had a positive effect on the activity of the preparation. However, the activity was decreasing gradually in the consecutive cycles. Therefore, the immobilization technique was slightly modified: glutaraldehyde was added to the alginate solution before mixing it with the enzyme, hereby the final glutaraldehyde concentration was 0.3%. After homogenization of three-component system gel beads were obtained from the solution by drop formation into CaCl_2 . Then the activity was measured similarly. At pH=3 and 4 the beads were swollen in this case, as well, they lost their mechanical stability, thus the activities were measured at neutral pH and in pH=5 buffer. Then the results were compared (Fig. 1 and Fig. 2).

It was found that the preparation has higher activity at acidic buffer in both cases (as it was expected) and the modified process improved the stability of the preparation and the activity loss was slower than in the former experiments. However, the added glutaraldehyde resulted in 15 - 20 % lower initial enzyme activity.

3.2 Immobilization on oak chips

The amount of enzyme required to saturate different amounts of carrier was examined. The filtrate generated during the immobilization process was not suitable for protein measuring, since the soluble pigment derived from oak chips interfered with spectrophotometric detection. The optimal enzyme / carrier ratio was determined on the basis of the urea eliminated. The initial urea concentration was 3000 ppm in 0.05 M pH=5 buffer. After 10 cm³ substrate addition the samples was shaken (120 rpm) at 37 °C for 30 minutes. Then the concentration of urea was measured. The data showed that the amount of eliminated urea was moved along a saturation curve as a function of amount of oak chips and 100 mg acid urease covalently bond on 500 mg carrier proved to be optimal, as it can be seen in Fig. 3.

It was observed in case of immobilization in alginate that the beads were swollen at lower acidic pH. Therefore, the system could not be evaluated after 2 - 3 cycles. Thus the stability of acid urease immobilized on oak chips was also examined at pH=3, 4 and 5 values. The measurements were carried out as it was described above and repeated substrate renewal was applied. This method has resulted in the system remained stable also at acidic pH values (Fig. 4).

After an initial decrease the immobilized enzyme preparation was able to maintain its urea elimination activity at nearly constant level for 20 days and acidic medium has not caused the loss of mechanical stability. Thereby, the preparation could be used in alcohol fermentation industry.

3.3 Comparison

It can be established that both enzyme immobilization methods were successfully implemented and the preparations were able to eliminate urea from model solutions with good efficiency.

In case of urease encapsulated in alginate beads relatively high initial activity values – about 800 U g⁻¹ – were obtained at pH=5. However, in distilled water medium the activity was significantly reduced (more than 50 %). The urea elimination capacity was decreased near

steadily with an average value of 5.4 U day⁻¹ at pH=5 and 5.0 U day⁻¹ at neutral pH, respectively. Furthermore, swollen and unstable beads were formed as a result of immobilization processes, which were carried out under more acidic conditions. This behaviour is disadvantageous and makes difficult to use the preparation in the alcohol fermentation. After modification with glutaraldehyde considerable decrease was observed in point of activity-loss rate, down to 2.2 U day⁻¹ at pH=5 and 2.7 U day⁻¹ at neutral pH. Nevertheless, the initial specific activity was lower due to the treatment.

The oak chips as enzyme support proved to be favourable in most aspects. The properties of the oak chips and cross-linking method made it possible to prepare an immobilized enzyme-preparation with outstanding storage stability and effective urea elimination capacity. After the initial period – 2 days – the activity decrease was reduced from 1.1-1.6 U day⁻¹ to lower than 0.1 U day⁻¹ then remained at this value even after 20 days (Fig. 5).

To study the rate-limiting (bottle-neck) step relaxation times of the reaction (t_R) and the diffusion (t_D) were determined by using the Yadav method (Table 1). It seems that the value of t_D is much lower thus the diffusion rate is higher than the reaction rate. Consequently the presence of diffusive inhibition could be excluded, the rates measured in the enzymatic process can be considered as the real reaction rates.

Although the amount of eliminated urea was less than in case of immobilization in alginate beads, the results of the measurements bring the applicability at lower pH values into prominence, namely the use under acidic conditions considered to be a key factor in terms of practical application. It should be also taken into account that wine industry materials and oak chips could be well fit together.

4. Conclusion

In this work different methods for preparation of immobilized biocatalyst for urea elimination were presented. In case of the encapsulation technique in alginate – regarded as traditional – the enzyme activity was negatively influenced by the addition of glutaraldehyde, the total activity decrease was 15 – 20 %. However, a cross-linked structure was formed in addition to

the alginate Ca^{2+} bindings which increased the operating duration of the enzyme. It was a disadvantage in terms of usability that the system was stable near neutral pH values only.

In case of the acid urease enzyme immobilization on oak chips high stability could be observed at acidic pH values, as well, and the urea elimination capacity loss of the preparation was only about 10 % even after 20 days. The measurements were confirmed that the enzyme activity is greater under acidic conditions. The optimal pH value of the operation was pH=3. Thus the acid urease immobilized by this method can be advantageously used e. g. in wine industry to prevent the formation of carcinogenic compounds.

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References

- Andrich, L., Esti, M., Moresi, M., 2010. Urea degradation kinetics in model wine solutions by acid urease immobilised onto chitosan-derivative beads of different sizes. *Enzyme and Microbial Technology*, 46, 397-405.
- Andrich, L., Esti, M., & Moresi, M., 2009. Urea degradation in model wine solutions by free or immobilized acid urease in a stirred bioreactor. *Journal of Agricultural and Food Chemistry*, 57, 3533-3542.
- Boross, L., Sisak, Cs., Szajáni, B., 2008. Solid-phase biocatalysts. Academic Press, Budapest.
- Bortone, N., Fidaleo, M., Moresi, M., 2012. Immobilization/stabilization of acid urease on Eupergit® supports. *Biotechnology progress*, 28, 1232-1244.
- DeGroot, A.R., Neufeld, R.J., 2001. Encapsulation of urease in alginate beads and protection from α -chymotrypsin with chitosan membranes. *Enzyme and Microbial Technology*, 29, 321-327.
- Francis, P. S., 2006. The determination of urea in wine – a review. *Australian Journal of Grape and Wine Research*, 12, 97-106.
- Krajewska, B., Leszko, M., Zaborska, W., 1990. Urease immobilized on chitosan membrane: preparation and properties. *Journal of Chemical Technology and Biotechnology*, 48, 337-350.
- Li, H.J., Cao, Z.K., Gao, J., Yu, J.Y., Liu, G.H., 2011. Immobilization of Acid Urease. *Chemical Industry and Engineering*, 4, 015.
- Martinsen, A., Skjåk-Bræk, G., & Smidsrød, O., 1989. Alginate as immobilization material: I. Correlation between chemical and physical properties of alginate gel beads. *Biotechnology and Bioengineering*, 33, 79-89.
- Matsumoto, K., 1992. Removal of urea from alcoholic beverages by immobilized acid urease, in: *Industrial Application of Immobilized Biocatalysts*. Marcel Dekker Inc., New York· Basel· Hong Kong, pp. 255-273.
- Mirvish, S.S., 1969. The carcinogenic action and metabolism of urethan and N-hydroxyurethan. *Advances in Cancer Research*, 11, 1-42.
- Nettleship, A., Henshaw, P.S., Meyer, H.L., 1943. Induction of pulmonary tumors in mice with ethyl carbamate (urethane). *Journal of the National Cancer Institute*, 4, 309-319.

- 1 Pannier, A., Oehm, C., Fischer, A.R., Werner, P., Soltmann, U., Böttcher, H., 2010.
2 Biodegradation of fuel oxygenates by sol-gel immobilized bacteria *Aquicola*
3 *tertiaricarbonis* L108. Enzyme and Microbial Technology, 47, 291-296.
4
5 Pithawala, K., Mishra, N., Bahadur, A., 2010. Immobilization of urease in alginate, paraffin
6 and lac. Journal of the Serbian Chemical Society, 75, 175-183.
7
8 Schlatter, J., Lutz, W.K., 1990. The carcinogenic potential of ethyl carbamate (urethane): risk
9 assessment at human dietary exposure levels. Food and Chemical Toxicology, 28, 205-
10 211.
11
12 Sogg, D., 2002. Oak flavorings. The Wine spectator, 27, 95-97.
13
14 Sungur, S., Elcin, M., Akbulut, U., 1992. Studies on immobilization of urease in gelatin by
15 cross-linking. Biomaterials, 13, 795-800.
16
17 Yadav, G.D., Devi, K.M., 2002. Enzymatic synthesis of perlauric acid using Novozym 435.
18 Biochemical Engineering Journal, 10, 93-101.
19
20
21
22
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Table 1 Relaxation times for enzymatic reaction and diffusion in case of various immobilized preparations.

Acid urease immobilization technique	t_R (s)	t_D (s)
Encapsulation in alginate beads (pH = 5)	3776.2	586.9
Encapsulation in alginate beads and glutaraldehyde cross-linking (pH = 5)	4408.1	586.9
Immobilization on oak chips (pH = 3)	8852.5	1843.5

Figure 1

Fig. 1. Eliminated urea values in distilled water and buffer (pH=5) as a function of time.

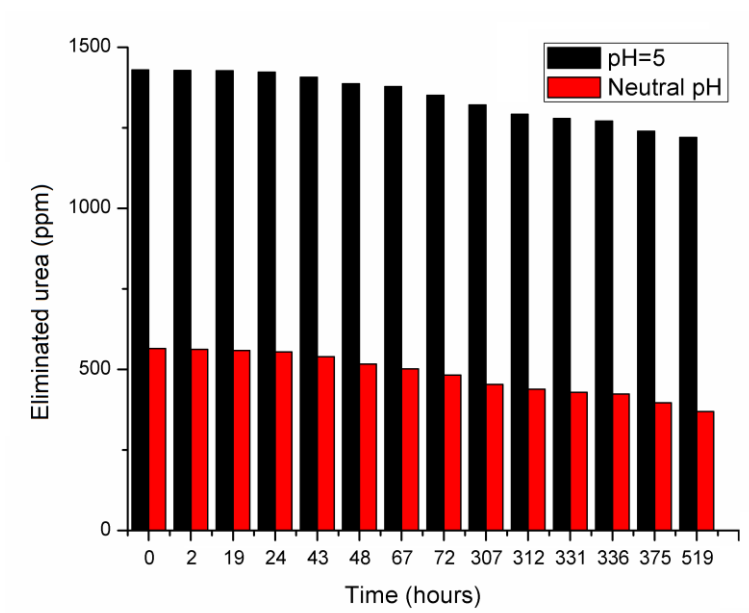


Figure 2

Fig. 2. Eliminated urea values in distilled water and buffer (pH=5) as a function of time in case of preparation modified by glutaraldehyde.

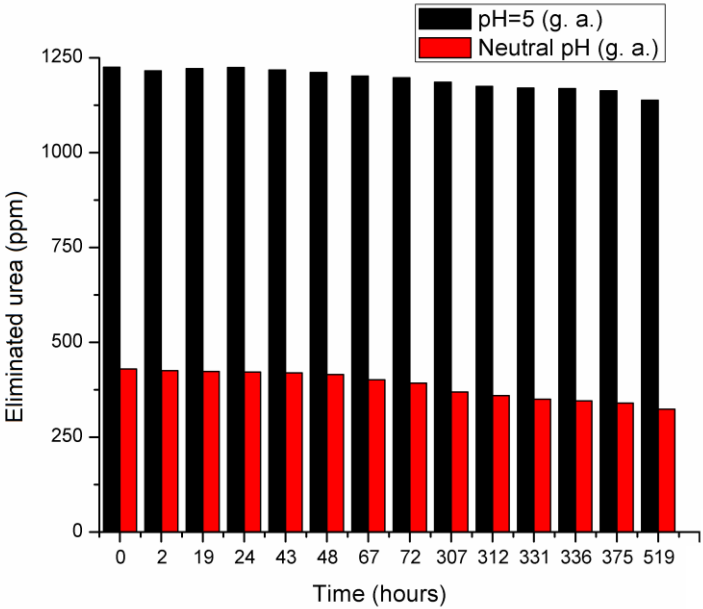


Figure 3

Fig. 3. Eliminated urea as a function of amount of oak chips carrier – determination of the optimal enzyme / carrier ratio.

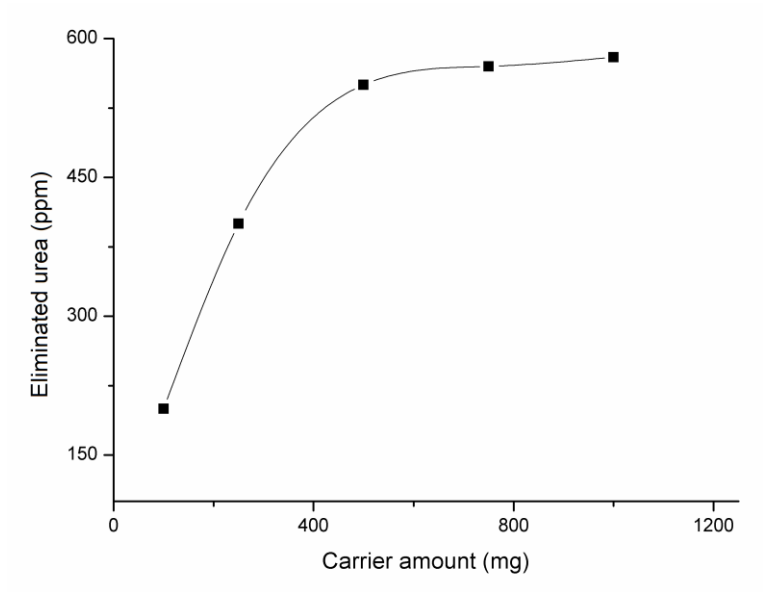


Figure 4

Fig. 4. Urea elimination capacity of the preparation as a function of time at various pH values.

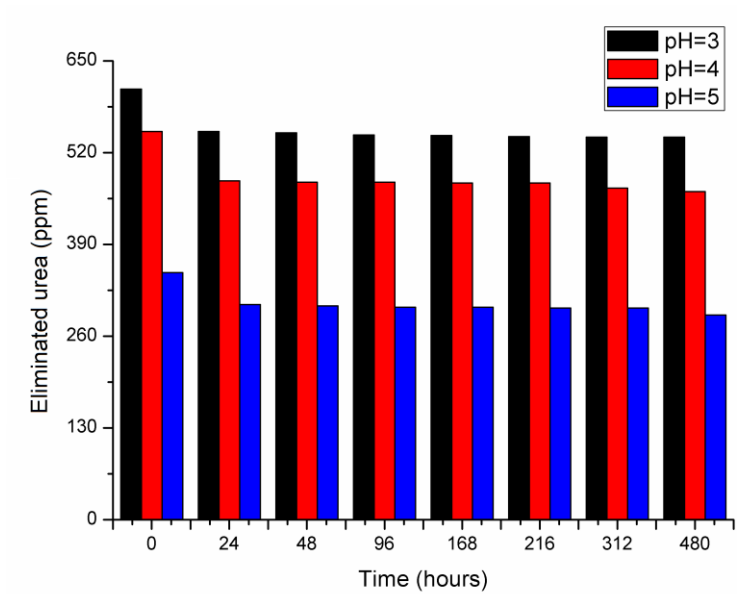


Figure 5

Fig. 5. Activity decrease as a function of time in case of oak chips-supported preparation.

