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MANUFACTURE OF ACID UREASE BY *LACTOBACILLUS FERMENTUM* FERMENTATION

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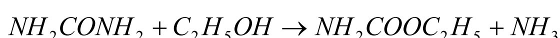
Ethyl carbamate can be found in fermented foods and alcoholic beverages, such as sake, beer and wine. It is considered as a carcinogenic compound for some animals, and probably carcinogenic to humans, as well. Ethyl carbamate is formed by a chemical reaction of ethanol and urea. The urea content should be reduced to less than 1 mg/l to prevent the formation of ethyl carbamate. Urease is an enzyme, which hydrolyzes urea into ammonia and carbon dioxide.

The acidic condition of fermented beverages is optimal for these types of ureases, which show high activity at low pH. Acid urease is an intracellular enzyme produced by Gram positive bacteria like *Lactobacillus fermentum*. It is commercially not easily available, hence it has to be produced by fermentation, extracted from the bacteria and purified properly.

Keywords: acid urease, *Lactobacillus fermentum*, fermentation

Introduction

Ethyl carbamate (urethane, EC) is a naturally occuring component in all fermented foods (soy sauce, yoghurt, bread) and alcoholic beverages (sake, wine, beer, brandy), being spontaneously produced by the reaction between urea and ethanol.



$$NH_2CONH_2 + C_2H_5OH \rightarrow NH_2COOC_2H_5 + NH_3$$

EC has been known to be carcinogenic, teratogenic and mutagenic. Therefore, removal of urethane or prevention of its formation from alcoholic beverages is an emerging problem all over the world [1]. As large amounts of ethyl carbamate were found in brandy, sake and distilled spirits, some governments established guidelines limiting the amount of EC in alcoholic beverages *(Table 1)*.

Table 1: Survey of ethyl carbamate levels (μg/l) in alcoholic beverages

Product	Range
Beer	0–5
Grape wine	0–10
Distilled spirits	10–6000
Sake	10–380
Fruit brandy	10–22000

In Hungary the maximum concentration of EC is 1 mg/l.

The most important precursor of ethyl carbamate in alcoholic beverages is assumed to be the urea produced during the metabolism of arginine by yeast. Ethanol is produced during the beverages fermentation, it reacts with urea and ethyl carbamate is formed [2]. Other precursors to the formation of ethyl carbamate are citrulline and carbamyl phosphate [3].

A lot of methods for decreasing urea level, hence preventing ethyl carbamate were tried by many scientists [4-6]. Possible methods of preventing ethyl carbamate formation in alcoholic beverages are shown in *Table 2* [7].

In our work the opportunities of enzymatic removal of urea by urease was studied.

Table 2: Possible way for preventing ethyl carbamate formation in alcoholic beverages

❖ Decreasing urea − Selection or breeding of yeast − Control of fermentation process − Utilization of urease − Utilization of artificial urease ▪ Adsorption site: chain of polyoxyethylene ▪ Active site: imidazole – COOH-Ni
❖ Control of pasteurization and storage of alcoholic beverages
❖ Utilization of urethanase: *Citrobacter sp.*

Acid urease

Urease enzyme *(Fig. 1)* is mainly found in the jack bean (*Canavalia ensiformis*). Its maximum activity is at pH 6.5–7.5. Therefore, urease with an optimum pH on the acid side (acid urease) was developed, because the pH of alcoholic beverages is low (about pH 4.4 and 3.2, respectively).

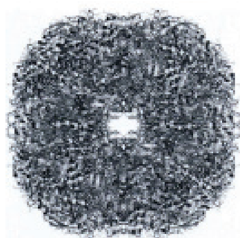


Figure 1: Urease enzyme

It has been known that urease hydrolyses urea into carbon dioxide and ammonia:

$$CO(NH_2)_2 + 2H_2O \rightarrow 2NH_3 + CO_2$$

Urease with an optimum pH was first found in *Lactobacillus sp.* from the rat gastrointestinal tract, *Lactobacillus fermentum* isolated from intestinal anaerobes in rat [9]. Urease from *Lactobacillus fermentum* was partially purified, characterized, and named acid urease by Takebe and Kobashi [10]. Acid urease was found in several *Lactobacillus, Streptococcus, Escherichia, Staphylococcus, Morganella, Bifido-bacterium, Arthrobacter* and *Zoogloea* species [9], and their optimal pH values are shown in *Table 3*.

Table 3: Optimal pH of several types of acid urease [8]

Optimal pH	Type of acid urease
pH=3	Acid urease from *Lactobacillus reuteri*
pH=3	Acid urease from *Lactobacillus fermentum*
pH=5	Acid urease from *Streptococcus bovis*
pH=4	Acid urease from *Streptococcus mitior*
pH=4	Acid urease from *Streptococcus salivarius*
pH=5	Acid urease from *Lactobacillus ruminis*

Bacteria and cultivation

Lactobacillus fermentum (Fig. 2) belongs to Gram-positive species of bacteria in the genus *Lactobacillus*. It is an acid urease producer strain [11].

Figure 2: Lactobacillus fermentum

L. fermentum was grown at 26 °C for 48 hours in a medium comprising 2% glucose, 0.5% yeast extract, 1% meat extract, 1% peptone, 0.1% TWEEN 80, 0.5% sodium acetate, 0.2% sodium citrate, 0.2% K_2HPO_4, 0.02% $MgSO_4{\cdot}7H_2O$, 0.005% $MnSO_4{\cdot}7H_2O$.

Methods for cell crushing

Cell crushing can be carried out from either the whole broth or the mass separated by filtration or centrifuge.

Lactobacillus fermentum has thick periplast (15–50 nm), consists of peptidoglucan, polysaccharide and teichoic acid *(Fig. 3)*.

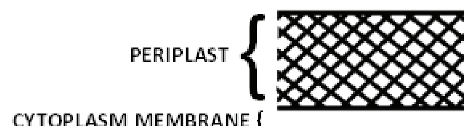


Figure 3: Periplast of Gram-positive bacterium [12]

Ultrasonic waves, extraction with surfactant solutions, salts and enzyme solutions can be applied for the cell crushing [12].

The cell crushing method has an optimal time course, the yield is getting worse after a certain time period *(Fig. 4)*.

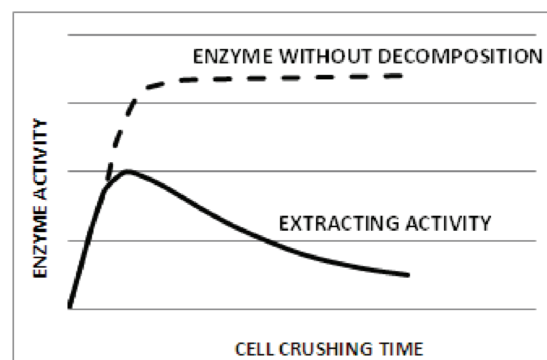


Figure 4: Relationship between cell crushing and enzyme activity

Enzyme solutions

Researchers have identified numerous enzymes that are able to crush periplast compound and the chemical bond *(Table 4)*.

Table 4: Specific enzymes for cell crushing

Microorganism	Enzyme
bacteria	lysozyme
yeasts	mannanase
mildews	chitinase, cellulase
algas	cellulase

During the enzymatic lysis not only the proper enzyme should be found, but its application method should be optimized, as well. Better results generally can be achieved by multi-enzyme. The advantage of the enzyme treatment is specificity, other compounds, bonds are not degraded. The lytic acting enzymes are expensive thus their applications are limited [12].

Determination of urea concentration

The methods for the determination of urea can be classified into three categories, based on colour-forming reactions, enzymatic hydrolysis and chromatographic separations.

Colour-forming reactions

These are non-enzymatic spectrophotometric analysis methods, where the spectrophotometric agent reacts with samples and generates a colourful compound. P-dimethylamino-benzaldehyde *(Fig. 5)*, butane-2,3-dione and 1-phenyl-1,2-propanedione-2-oxime are applied as reagents [13].

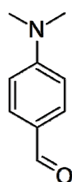


Figure 5: Para-dimethylamino-benzaldehyde

Enzyme catalysed hydrolysis

The enzymatic methods include hydrolysis of urea by urease then ammonia formed is measured. Test kit is available and used by numerous researchers. The ammonia measurement method is based on the reaction between ammonia and 2-oxoglutarate. NADH is reduced by ammonia and the decrease of it – monitored by absorbance at 340 nm – is proportional to the amount of ammonia therefore the urea concentration can be determined in the original sample [13].

Chromatographic separation

High-performance liquid chromatography (HPLC) and gas chromatography (GC) are used to separate and quantify many components in alcoholic beverages. HPLC with fluorescence detection and GC with capillary column are applied for the determination [13-15].

Determination of acid urease activity

During the determination of acid urease activity 100 µl 0.6% urea solution was added to the mixture of 0.5 ml of enzyme solution and 2.5 ml of 0.1 M pH 4.0 citrate buffer containing 20% ethanol, and it was mixed. After exactly 30 minutes at 37 °C 4 ml of 10% TCA solution was added to stop the reaction. Under these conditions, 1 unit is defined as the activity which liberates 1 µmole of ammonia per minute at an initial reaction [16].

Colour-forming reaction was applied for the determination of urea concentration. The reagent was P-dimethylamino-benzaldehyde. In this method 5 ml of urea solution and 5 ml of P-dimethylamino-benzaldehyde solution were mixed. After 10 minutes at room temperature the samples were measured at 440 wavelength in the spectrophotometer. [17]

Method of the cell crushing

One liter of cultured medium (culture broth) was centrifuged to obtain 7.0 g of wet cells, which were suspended in 10 mM phosphate buffer (pH 7.0) to make 10 ml suspension. To this suspension 10 mM phosphate buffer (pH 7.0) containing 0.02 g of Triton X-100 and 2 mg of lysozyme were added. After agitation the mixture was allowed to stand for 3 days at 30 °C. Cell residue was removed by centrifugal separation to obtain a crude enzyme solution.

After it the supernatant was precipitated by ice-cooled ethanol to adjust final ethanol concentration to 60%, and the mixture was subjected to centrifugal separation. The resulting filter cake was dissolved in 10 mM phosphate buffer (pH 7.0) and the solution was dried [18].

Results and Discussion

Ethyl carbamate is a carcinogenic, teratogenic and mutagenic component in alcoholic beverages and is chemically produced from urea and ethyl alcohol.

Acid urease was developed for removal of EC or prevention of its formation in beverages, and it was found in several bacteria.

In this work acid urease was manufactured from *Lactobacillus fermentum*. Our aim was to prepare acid urease with higher quantity and activity. 0.223 g 7 U/g crude dried enzyme was possible to prepare by cell crushing using lysozyme and Triton X-100 then precipitation of the supernatant by ethanol.

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