PURIFICATION AND CHARACTERIZATION OF DECARBOXYLASE FROM A HALOPHILIC MICROORGANISM WITH POTENTIAL IN BIOTRANSFORMATION OF WASTE GLYCEROL FROM BIODIESEL INDUSTRY

NEAGU Simona, COJOC Roxana, GOMOIU Ioana, TUDORACHE Mădălina, ENACHE Mădălin

Abstract. The present study reveals the influence of the culture medium composition on decarboxylase production by a strain of halotolerant bacteria isolated from the Letea salt lake in the Danube Delta, namely strain LN 1-17, the purification of the enzyme obtained on the culture medium that offers optimal conditions for its production, as well as the enzyme characterization. The results of this study confirm that the investigated strain harbors the capacity to produce lysine decarboxylase. Related to the influence of culture medium composition the best result was recorded on MH medium supplemented with lysine and casamino-acids. The purification of the enzyme by precipitation with acetone followed by gel filtration was considered appropriate due to the increase of the enzymatic specific activity even if it has led to a decrease in the protein content. The biochemical characterization of the enzyme showed an optimal activity at neutral pH (7), in the absence of NaCl and at a temperature of 60^0C.

Keywords: decarboxylase, halophilic microorganisms, waste glycerol, biodiesel.

Rezumat. Purificarea şi caracterizarea decarboxilazei de la un microorganism halofil cu potenţial în biotransformarea glicerolului rezidual din industria biodiesel. Studiul de faţă se referă la influenţa compoziţiei mediului de cultură asupra producerii de decarboxilaze de către o tulpină bacteriană halotolerantă izolată din lacul sărat Letea din Delta Dunării, respectiv tulpina LN 1-17, la purificarea enzimei obţinute pe mediu de cultură care oferă condiţii optime pentru obţinerea acesteia precum şi la caracterizarea enzimei. Rezultatele acestui studiu confirmă că tulpina investigată are capacitatea de a produce lizin decarboxilază. În ceea ce priveşte influenţa compoziţiei mediului de cultură, cele mai bune rezultate au fost obţinute pe mediul de cultură MH suplimentat cu lizină şi acizi cazaminici. Purificarea enzimei prin precipitarea cu acetonă, urmată de gel filtrare, chiar dacă a condus la scăderea conţinutului de proteină este adecvată, deoarece conduce la creşterea activităţii specifice a enzimei. Caracterizarea biochimică a enzimei a arătat că aceasta are activitate optimă la pH neutru (7), în absenţa NaCl şi la 60^0C.

Cuvinte cheie: decarboxilaze, microorganisme halofile, glicerol rezidual, biodiesel.

INTRODUCTION

Waste glycerol is the by-product obtained in large amounts in the manufacturing process of biodiesel (KALIA et al., 2016; NEAGU et al., 2018b in press). This product began to represent a problem related to the ratio efficiency/price of the biodiesel industry processes. In spite of the fact that the glycerol molecule by its structure has huge application, the waste glycerol has no value for the synthesis industry due to the contained impurities like methanol, water, salts, soaps (HIRSCHMANN et al., 2005). On the other hand, the purification of waste glycerol is not a feasible alternative. The lack of proper management for waste glycerol entails the lowering of the biodiesel industry's interest due to high production costs and an increased environmental pollution by large glycerol stocks (NEAGU et al., 2018).

Residual glycerol is the most important by-product in biodiesel production from biomass (DASARI et al., 2005). Lately, worldwide biodiesel production has substantially grown. As a direct consequence, residual glycerol (unpurified) has been produced in quantities that will soon outweigh the current market requirements, constituting a serious environmental problem by its storage. The idea of producing an enormous amount of residual glycerol is automatically associated with enormous losses of energy and material resources. Moreover, a hyperproduction of glycerol may result in a significant decrease in its price. Another aspect of the problem of residual glycerol is that its applicability is reduced compared to that of purified glycerol. This is due to numerous impurities found in the residual glycerol matrix (i.e. water, salts, soaps, etc.) (HIRSCHMANN et al., 2005). As a consequence, residual glycerol is used in industry only for the production of dried pet food (LEONETI et al., 2012). In literature, attempts are made to use residual glycerol but in a mimic laboratory form, i.e. pure glycerol contaminated with supposed impurities, for developing the glycerol industry on the residual glycerol branch by designing and applying new synthesis technologies (YUAN et al., 2010; LAURIOL-GARBEY et al., 2011).

Bioconversion of residual glycerol can be accomplished through several paths like its transformation into glycerol carbonate by carbonylating, glycidol and glycerol carbonate compounds which, under the action of decarboxylase, can be converted to polyglycerol (TUDORACHE et al., 2017).

Decarboxylases are produced by most microorganisms in response to their development in acidic environments, by acting to protect the microbial cell from the noxious action of acid media (GALE & EPPS, 1944; ALVAREZ-ORDÓÑEZ et al., 2010). These are enzymes of the carboxylases class, subclass E.C. 4.1.1, which catalyze the removal of the carboxyl group from an organic compound with carbon dioxide and proton forming. In bacteria, the amino acids decarboxylases appear to be either inducible or constitutive. On the other hand, if a multiple copy of the gene which codify the synthesis of lysine decarboxylase are present, the level of enzymatic activity increase (VENTOSA et al., 1989; KIKUCHI et al., 1997).

The purpose of this study was to analyze the influence of the culture medium composition on decarboxylase production by a strain of halotolerant bacteria isolated from Letea salt lake in the Danube Delta, to purify the enzyme obtained on the culture medium that offers optimal conditions for its production, as well to characterize the enzyme.

MATERIALS AND METHODS

The influence of the culture medium composition on decarboxylase production. In a first step, it was attempted to stimulate the production of lysine-decarboxylase by the halotolerant bacterial strain. Three variants of the culture medium for enzyme biosynthesis were used. Thus, the first variant of the culture medium (GSM) was prepared starting from 3 stock solutions: solution A containing 4g Na_2HPO_4 x $12H_2O$, 5g KH_2PO_4, dissolved in 100 mL distilled water; solution B containing 10g NH_4Cl, 5g NaCl, 4.1g $MgSO_4$ x$7H_2O$, dissolved in 100 mL distilled water; solution C formed by 8g glucose, 4g lysine and 8g casamino-acids dissolved in 315 mL distilled water.

Solutions A and B were sterilized by autoclaving at 121^0C for 20 minutes, and solution C was sterilized by filtration on a Millipore membrane with 0.22 μm pore size. A volume of 20 mL from solution A was mixed in sterile conditions with 1.1 mL solution B and 78,9 mL solution C (WANDA LU & MALLETTE, 1970). A negative control of the three stock solutions was also carried out without adding the casamino-acids to the culture medium, which would contribute to the stimulation of enzyme synthesis. The second variant of the culture medium contained (g/L): yeast extract 10, NaCl 100, $MgCl_2$x$6H_2O$ 7, $MgSO_4$x$7H_2O$ 6, $CaCl_2$x$2H_2O$ 0.36, KCl 2, $NaHCO_3$ 0.06, NaBr 0.026 (MH medium, VENTOSA et al., 1972), supplemented with cu 1% lysine and 2.5% casamino-acids.

The third variant of the culture medium was MH medium, supplemented with 1% lysine. For these culture medium variants, a volume of 10 mL of bacterial inoculum was used, whose optical density at 660 nm was 0.4. Bacterial cultivation was performed at 30°C, 140 rpm. After 24 hours, the strain grew on all culture media, except for the negative control of the first variant of culture medium. The decarboxylase activity was determined after 24h and 72h of incubation of the strain in the tested culture media.

Decarboxylase activity assay. Decarboxylase activity has been determined by the Lenhoff method (PHAN et al., 1982). Each test tube contained: 20 μL enzymatic extract, 256 μL of 10 mM phosphate buffer, pH 7 and 24 μL of 100 mM lysine solution. The mixture was incubated for 30 minutes at 30°C. The reaction was stopped with 300 μL K_2CO_3, for five minutes at 30°C. After this step, 300 μL of 20 mM 2,4,6-trinitrobenzene sulfonic acid (TNBS) were added. The obtained reaction product (cadaverine) was extracted with 600 μL of toluene. The mixture was vortexed for 1 min and the final product separated into two phases: an orange aqueous phase and an organic phase, colorless to yellow. Aliquots of 200 μL of the organic layer were taken and absorbance was determined at 340 nm with toluene as blank. The amount of cadaverine released per time unit, determined at 340 nm, is a measure of lysine-decarboxylase activity. Catalytic activity was expressed as μmoles/min/mL.

Enzyme purification. After the growth of the bacterial culture and the enzyme biosynthesis, the culture liquid was centrifuged at 9500 g for 10 minutes at 4°C. The supernatant was removed, and the biomass was stored at -80 °C until the next step. The cells were treated with 40 mL of 0.9% NaCl solution and centrifuged. The washed cells were suspended in 30 mL of 0.02 M phosphate buffer, pH 7. Ten mL of this suspension were sonicated (15 repeats/5sec). The resulting mixture was centrifuged at 4°C for 20 minutes, 4000g, to remove cell debris. The obtained supernatant was considered crude enzyme extract. A volume of 10 mL was subjected to partial purification by precipitation with 80% acetone. This purification step was carried out at negative temperatures (-5°C), the ratio of acetone: enzymatic extract being 2: 1 (v/v). Acetone was added gradually, under continuous stirring, taking care that the temperature shall exceed -2°C, after which it was left in the refrigerator for 24h. The obtained precipitate was separated by centrifugation at 4°C, 9500 rpm, 20 minutes. The obtained acetone powder was stored and dried at room temperature for 60 minutes, then suspended in a volume of 10 mL of 50 mM Tris-HCl buffer, pH 7.5.

The resulting protein extract was purified by gel filtration in 1 mL aliquots. The work protocol required, in a first step, the preparation of the gel filtration column by washing it with MilliQ water and 96% ethanol and charging it with 2 mL of gel obtained by hydrating 2 g of BioGel P-Biorad copolymer P100 with 50 mL of MilliQ water for 12h at 20°C. The equilibration was performed with 0.05 M Tris-HCl buffer pH 7.5, containing 0.003 M EDTA and 0.001 M β-mercaptoethanol. Elution was performed with 0.1M, 0.2M, 0.5M and 1M Tris-HCl, pH 7.5, in three repetitions for each concentration. Protein concentration (Lowry method – LOWRY et al., 1951) and enzymatic activity were determined at each step by the method described by Lenhoff, with some modifications (KIM et al., 2015). The decarboxylase activity was expressed as μmoles/min/mg of protein.

Biochemical characterization:

The pH influence on lysine decarboxylase activity. The decarboxylase activity of the purified extract was determined in reaction media with pH values between 4-10, the other incubation conditions remaining constant (30°C, 0% NaCl, 100 mM lysine).

The influence of temperature on lysine decarboxylase activity. In order to determine the optimal reaction temperature of the purified protein extract, it was incubated at various temperature values between 4-60°C, the other incubation conditions remaining constant (pH 7, 0% NaCl, 100 mM lysine).

The influence of NaCl concentration on lysine decarboxylase activity. In order to determine the optimum concentration of NaCl for the catalytic activity of the protein extract, the salt concentration was varied from 0 to 3 M NaCl in the reaction medium.

RESULTS AND DISCUSSION

The influence of culture medium composition on the decarboxylase production. Following the qualitative study, which aimed to identify strains of halophilic/halotolerant bacteria able to synthesize lysine-decarboxylase, the strain LN1-17 was selected to obtain a purified enzyme preparation and to establish the optimal catalytic activity parameters. The strain was isolated from Letea Salt Lake located in the Danube Delta, whose salinity is influenced by environmental conditions ranging from 7 g/L in springtime to 32 g/L during summer and autumn (NEAGU et al., 2018b in press).

The preliminary characterization of the strain revealed a large yellow-orange, glossy, flat, reniform colony, Gram negative staining, short, isolated rods. The strain grew between 0-4M NaCl with optimal from 0 to 3M. The strain was tested for decarboxylase production following the method previously described (NEAGU et al., 2016) and positive results were recorded. In order to estimate the influence of culture medium composition on decarboxylase production, the strain was growth on GSM and MH media with various chemical compositions as described in materials and methods and the enzymatic activity has been evaluated at 24 and 72 hours of cultivation.

The data obtained showed that the highest decarboxylase activity was obtained 24 hours after the inoculation in the second culture variant (MH medium with lysine addition and casamino acids – Table 1). Thus, for the subsequent steps of enzymatic biosynthesis, purification and biochemical characterization of lysine decarboxylase, MH culture medium supplemented with 1% lysine and 2.5% casamino acids was used.

Table 1. Decarboxylase activity of the strain LN1-17 after 24h and 72h of growth on different culture media; I (GSM) – medium with glucose, salts, lysine and casamino-acids; II – MH medium supplemented with lysine and casamino-acids; III- MH medium supplemented with lysine.

Culture medium variant / Enzyme activity (µmoles/min/mL)	I (GSM24h)	I (GSM48h)	II (24h)	II (72h)	III (24h)	III (72h)
LN1-17	0.0043	0.0038	0.012	0.0076	0.0085	0.0058

Enzyme purification. The enzyme purification protocol has been detailed in materials and methods area. In a first step of the method the enzyme has been purified with 80% cold acetone at negative temperature and in a final step a gel filtration using BioGel P-Biorad P100 gel for column preparation, was applied.

The results presented in Table 2 showed that the amount of enzyme decreased in each step of the purification starting from 13,5 mg/mL in the first step until to 0,27 mg/mL in last step of the gel filtration. On the other hand, the specific activity of the enzyme increased up to 0.034 µmoles/min/mg (Table 2). The partially purified enzyme preparation was biochemically characterized by determining the optimal physicochemical parameters of catalytic activity.

Table 2. The purification steps of lysine-decarboxylase synthesized by the halotolerant bacterium strain LN1-17.

No.	Purification step	Volume (mL)	Protein content (mg/mL)	Specific activity (µmoles/min/mg)
1	Culture filtrate	100	13.5	0.0005
2	Acetone precipitation 80%	10	6.69	0.0008
3	Gel filtration	1	0.27	0.034

Biochemical characterization of the purified enzyme

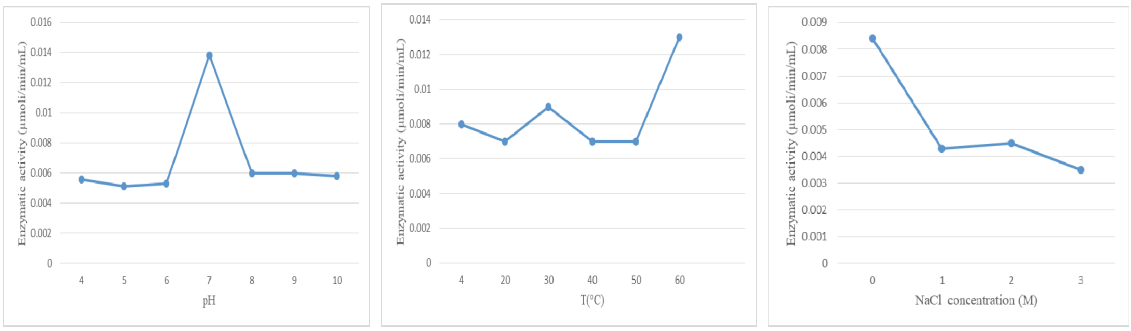
The pH influence on lysine decarboxylase activity. As presented in Table 3 and Fig. 1a, the partially purified decarboxylase exhibited catalytic activity at pH values between 4-10, with a maximum at pH 7.

Table 3. The decarboxylase activity of the purified protein extract, at different pH values (µmoles/min/mL).

pH / Strain	4	5	6	7	8	9	10
LN1-17	0.0056	0.0051	0.0053	0.0138	0.006	0.006	0.0058

The influence of temperature on lysine decarboxylase activity. From the data presented in Table 4 and Fig. 1b, the presence of catalytic activity is observed in the temperature range 4-60˚C, the optimal activity being observed at 60˚C.

The influence of NaCl concentration on lysine decarboxylase activity. It was found that the decarboxylase synthesized by strain LN1-17 showed catalytic activity at values of 0-3 M NaCl concentration, the activity being maximum at the concentration of 0 M NaCl, which confirms the halotolerant character of the enzyme that is correlated with the growth of the bacterial strain (Table 5 and Fig. 1c).



(a) (b) (c)

Figure 1. Influence of pH (a), temperature (b) and NaCl concentration (c) on the catalytic activity.

Table 4. The decarboxylase activity of the purified protein extract, at different values of temperature (µmoles/min/mL).

Temperature (°C) / Strain	4	20	30	40	50	60
LN1-17	0.008	0.007	0.009	0.007	0.007	0.013

Table 5. The decarboxylase activity of the purified protein extract, at different NaCl concentrations (µmoles/min/mL).

NaCl concentration (M) / Strain	0	1	2	3
LN1-17	0.005	0.0043	0.0045	0.0035

CONCLUSIONS

The results from this study evidence the capacity of the strain LN 1-17 to produce extracellular protein with lysin-decarboxylase activity. The enzymatic activity appears to be influenced by the composition of the culture medium, the best result being recorded on MH medium supplemented with lysine and casamino-acids. The purification of the enzyme by precipitation with acetone followed by gel filtration was considered appropriate due to the increase of the enzymatic specific activity even if it has led to a decrease in protein content. The biochemical characterization of the enzyme showed optimal activity at neutral pH (7), in the absence of NaCl and at 60^0C. The activity at increased temperature could be an advantage for using the enzyme in various industrial and biotechnological processes and for obtaining biosensors for monitoring technological processes at high temperature or monitoring parameters in extreme environments.

ACKNOWLEDGEMENTS

This work was supported by Program Partnerships in Priority Domains – PN II developed with the support of MEN-UEFISCDI, Project No. 273/2014, and the Project No. RO1567-IBB05/2018 from the Institute of Biology Bucharest of the Romanian Academy.

REFERENCES

ALVAREZ-ORDÓÑEZ A., FERNÁNDEZ A., BERNARDO A., LÓPEZ M. 2010. Arginine and lysine decarboxylases and the acid tolerance response of Salmonella typhimurium. *International Journal Food Microbiology*. Elsevier. Paris. **136**: 278-282.

DASARI M. A., KIATSIMKUL P. P., SUTTERLIN W. R., SUPPES G. J. 2005. Low-pressure hydrogenolysis of glycerol to propylene glycol. *Applied Catalysis A: General.* Elsevier. Paris. **281**: 225-231.

GALLE B. F. & EPPS M. R. H. 1944. Studies on bacterial aminoacids decarboxylases – l (+) lysine decarboxylases. *Biochemical Journal*. Cambridge University Press. London. **38**: 232-242.

HIRSCHMANN S., BAGANZ K., KOSCHIK I., VORLOP K. D. 2005. Development of an integrated bioconversion process for the production of 1,3-propanediol from raw glycerol waters. *Landbauforsch Volkenrode*. Johann-Heinrich-von-Thünen-Institut. Brunswick. **55**: 261-267.

KALIA V. P., PRAKASH J., KOUL S. 2016. Biorefinery for glycerol rich biodiesel industry waste. *Indian Journal of Microbiology*. Springer. New Delhi. **56**: 113-125.

KIKUCHI Y., KOJIMA H., TANAKA T., TAKATSUKA Y., KAMIO Y. 1997. Characterization of a second lysine decarboxylase isolated from *Escherichia coli. Journal of Bacteriology*. American Society for Microbiology. New York. **179**: 4486–4492.

KIM Y. H., SATHIYANARAYANAN G., KIM H. J., BHATIA S. K., SEO H. M., KIM J. H., SONG H. S., KIM Y. G., PARK K., YANG Y. H. 2015. A Liquid-Based Colorimetric Assay of Lysine Decarboxylase and Its Application to Enzymatic Assay. *Journal of Microbiology and Biotechnology*. The Korean Society for Microbiology and Biotechnology. Seoul. **25**: 2110-2115.

LAURIOL-GARBEY P., POSTOLE G., LORIDANT S., AUROUX A., BELLIERE-BACA V., REY P., MILLET J. M. M. 2011. Acid–base properties of niobium-zirconium mixed oxide catalysts for glycerol dehydration by calorimetric and catalytic investigation. *Applied Catalysis B: Environmental*. Elsevier. Paris. **106**: 94-102.

LEONETI A. B., ARAGÃO-LEONETI V., DE OLIVEIRA S. V. W. B. 2012. Glycerol as a by-product of biodiesel production in Brazil: Alternatives for the use of unrefined glycerol. *Renewable Energy*. Elsevier. Paris. **45**: 138-145.

LOWRY J. O. H., ROSEBROUGH N. J., FARR A. L., RANDAL R. J. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*. American Society of Biological Chemists Publisher. Maryland. **193**: 256-277.

NEAGU SIMONA, COJOC ROXANA, GOMOIU IOANA, ENACHE M. 2016. The production of lipases and decarboxylases by halophilic bacteria able to grow in the presence of waste glycerol. *Oltenia. Studii şi Comunicări. Ştiinţele Naturii*. Muzeul Olteniei Craiova. **32**: 137-142.

NEAGU SIMONA, COJOC ROXANA, TUDORACHE MĂDĂLINA, GOMOIU IOANA, ENACHE M. 2018a. The lipase activity from moderately halophilic and halotolerant microorganisms involved in bioconversion of waste glycerol from biodiesel industry. *Waste Biomass Valor*. Springer. Zurich. **9**: 187-193.

NEAGU SIMONA, COJOC ROXANA, ENACHE M., MOCIOIU O. C., PRECUPAS A., POPA V. T., GOMOIU IOANA. 2018b. Biotransformation of waste glycerol from biodiesel industry in carotenoids compounds by halophilic microorganisms. *Waste Biomass Valor*. Springer. Zurich. In press.

PHAN A. P. H., NGO T. T., LENHOFF H. M. 1982. Spectrophotometric assay of lysine decarboxylase. *Analles Biochemmical*. Elsevier. Paris. **120**: 93-197.

TUDORACHE MĂDĂLINA, NEGOI A., PÂRVULESCU V. I. 2017. Enhancement of the valorisation of renewable glycerol: The effects of the surfactant-enzyme interaction on the biocatalytic synthesis of glycerol carbonate. *Catalysis Today*. Elsevier. Paris. **279**: 71-76.

VENTOSA A., GARCIA M. T., KAMEKURA M., ONISHI H., RUIZBERRAQUERO F. 1989. *Bacillus halophilus* sp. nov., a new moderately halophilic *Bacillus* species. *Syst. Appl. Microbiol*. Springer. Berlin. **12**: 162-166.

WANDA LU W. W. & MALLETTE M. F. 1970. Colorimetric Assay for Lysine Decarboxylase in *Escherichia coli*. *Applied Microbiology*. American Society for Microbiology. New York. **19**: 367-369.

YUAN Z., WANG J., WANG L., XIE W., CHEN P., HOU Z., ZHENG X. 2010. Biodiesel derived glycerol hydrogenolysis to 1,2-propanediol on Cu/MgO catalysts. *Bioresource Technology*. Elsevier. Paris. **101**: 7088-7092.

Neagu Simona, Cojoc Roxana, Gomoiu Ioana, Enache Mădălin
Institute of Biology Bucharest of the Romanian Academy, Spl. Independentei 296, Sect. 6, P.O. Box 56-53, 060031, Bucharest, Romania.
E-mail: madalin.enache@ibiol.ro

Tudorache Mădălina
University of Bucharest, B-dul M. Kogalniceanu, no. 36-46, Bucharest-050107, Romania.

Received: March 31, 2018
Accepted: July 8, 2018