THE PRODUCTION OF LIPASES AND DECARBOXYLASES BY HALOPHILIC BACTERIA ABLE TO GROW IN THE PRESENCE OF WASTE GLYCEROL

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Abstract. Chemically, biodiesel is obtained from the trans-esterification of vegetable oil or animal fat, in the presence of alcohol, such as methanol or ethanol, generating glycerol as by-product. The aim of this work was to test the capacity of several halophilic microorganisms to grow on culture media supplemented with waste glycerol, derived from different sources (Mediaș and Slobozia), and to produced lipases and decarboxylases used as biocatalysts to convert residual glycerol to valuable compounds. From a total of 30 halophilic bacterial strains, isolated from Balta Albă, Buzău County, and Techirghiol salt lakes, a number of eight halophilic/halotolerant strains showed lipolytic activity. *Marinococcus halophilus* strain JCM 2472, a moderately halophilic bacteria, was used in this study for its capacity to synthesized extracellular lipases and decarboxylases.

Keywords: waste glycerol, halophiles, lipases, decarboxylases.

Rezumat. **Producerea de lipaze și decarboxilaze de către bacterii halofile capabile să se dezvolte în prezența glicerolului rezidual.** Biodiesel se obține prin reacția de trans-esterificare a uleiurilor vegetale sau a grăsimilor de origine animală, în prezența unui alcool, metanol sau etanol, cu generare de glicerol ca produs secundar. Scopul acestui studiu a fost de a testa capacitatea microorganismelor halofile de a se dezvolta în prezența glicerolului rezidual și de a produce lipaze și decarboxilaze utilizate ca biocatalizatori în conversia glicerolului rezidual la compuși valoroși. Dintr-un număr de 30 de tulpini de bacterii halofile izolate din probe de apă, prelevate din lacul sărat Balta Albă, județul Buzău și din lacul Techirghiol, au prezentat activitate lipolitică un număr de opt tulpini de bacterii halofile/halotolerante. *Marinococcus halophilus* JCM 2472, a fost selectată și utilizată în studiu datorită capacității acesteia de a sintetiza extracelular, lipaze și decarboxilaze.

Cuvinte cheie: glicerol rezidual, halofile, lipaze, decarboxilaze.

INTRODUCTION

The obtaining process of biodiesel consists in transforming triglycerides into fatty acid alkyl esters in the presence of alcohol, such as methanol or ethanol, and an acid or alkali catalyst, generating glycerol as a major by-product (PALLIGARNAI et al., 2007). The biodiesel, regarded as the fuel of the future, and an alternative of the fossil fuel, represents a nontoxic, biodegradable, and renewable source of combustible. It is estimated that the production of biodiesel will generate about 10% (w/w) glycerol as the main by-product (FANGXIA et al., 2012). The development of the biodiesel industry entails a surplus of waste glycerol considered as waste stream, being an inconvenience for the efficiency/costs ratio of biodiesel process. Taking this into account, it is necessary to convert waste glycerol to useful products. The studies reported in the literature revealed the possible valorisation of raw glycerol by involving different processes, such as direct application, chemical transformation, or microbial conversion (CHENG et al., 2013). Making a comparison between direct applications, which implies utilization of waste glycerol as a simple carbon source, chemical transformation with processes, which involve expensive metal catalysts, toxic intermediates, and low conversion rates, the microbial conversion of glycerol became a valuable and attractive application (CHENG et al., 2013).

Several microbial strains belonging to Enterobacteriaceae Family were involved in the fermentation of glycerol. *Escherichia coli*, considered as bacterial platform for the production of useful metabolites (CHENG et al., 2007), has the capacity to transform glycerol into ethanol, lactic, succinic or acetic acid, fatty acids omega-3 polyunsaturated, 1,2-propanediol (1,2-PDO), 1,3-propanediol (1,3-PDO) (YANG et al., 2012). Also, *Klebsiella*, *Citrobacter*, and *Clostridium* species present the ability to convert glycerol to 1,3-propanediol (1,3-PDO) (CHENG et al., 2007; METSOVITI et al., 2013; BIEBL et al., 1992). Some yeast and fungi species, such as *Yarrowia lipolytica*, or *A. niger* showed the potential to transform waste glycerol into citric acid (NICOL R.W. et al, 2012). The biocatalytic conversion of waste glycerol to valuable compounds, such as glycidol, or glycerol carbonate, using an enzymatic cocktail formed by lipases and decarboxylases, represents an attractive and environmentally friendly alternative (NEAGU et al., 2015).

The main goal of this work was to identify moderately halophilic microorganisms able to grow in the presence of waste glycerol as a carbon source and to select the strains able to synthesized extracellular lipases and decarboxylases involved in the conversion of substrate (waste glycerol) to value added product like glycerol carbonate and glycidol.

MATERIAL AND METHODS

Cultures and media

The halophilic bacterial strains used in this study were isolated from the saline lake Balta Albă, located in Romania, in Buzău-Brăila counties approximately 170 km East of Bucharest, and Techirghiol Lake, located near the Black Sea coast. A set of serial dilutions was made into sterile saline solution, and one millilitre from each water sample and dilution was distributed in drops onto Petri dishes. The molten agar media (around 50 – 55^{0}C) was poured and the

plates were incubated at 28^0C, for 7-10 days. The resulted colonies were counted and after that were purified by repeated streaking on 10% MH agar medium. The isolation media (MH) contained (g/L): NaCl - 100, $MgCl_2x6H_2O$ - 7, $MgSO_4x7H_2O$ - 9.6, $CaCl_2x2H_2O$ - 0.36, KCl - 2, $NaHCO_3$ - 0.06, NaBr - 0.026, glucose - 1, proteose peptone - 5, yeast extract - 10 (VENTOSA et al.,1989). The pH of the culture medium was adjusted to 7.0 – 7.2 before autoclaving. It was established the affiliation of halophilic strains to *Bacteria* or *Archaea* domain. The growth of halophilic strains on MH solidified medium containing deoxycholic acid sodium salt, a bile acid salt, at a concentration of 0.004%, or chloramphenicol 0.002% allowed the differentiation between halophilic bacteria and halophilic archaea. All tested strains grew on media with deoxycholic acid sodium salt and, thus, were considered halophilic bacteria. *Marinococcus halophilic* strain JCM 2472 (courtesy of dr. Takashi Itoh – Japan Collection of Microorganisms) was used in further experiments, and growth medium for the strain was R-AGAR, and contained (g/L): NaCl - 50, peptone - 10, casamino acids - 5, malt extract - 5, yeast extract - 5, beef extract - 2, $MgSO_4x7H_2O$ – 1, and Tween 80 – 0.05.

Waste glycerol source

The types of residual glycerol used in the experiments were represented by manufacturer suppliers: Mediaș and Slobozia sources. The growth ability of strains in the presence of waste glycerol was monitored at 660 nm with a UV/VIS spectrophotometer (BMG LABTECH FLUOStar Omega microplate reader).

Determination of extracellular lipase activity

Lipase activity of the bacterial cultures was screened qualitatively following the method described by BAHTNAGAR et al. (2005) with some modifications. The sterilized basal medium was supplemented with 1.25% olive oil (w/v) and the mixture was homogenized. The 0.001% rhodamine B (w/v) was added in medium at 50^0C, before pouring into plates. The lipase activity was tested at 0, 1.7, and 3M NaCl. The similar sized wells were cut in the solidified medium and a volume of 200 µl of bacterial culture was placed in each well, and incubated at 28^0C, for 48 hours. The colonies with an orange-red halo under UV light were considered positive.

Determination of decarboxylase activity

The production of decarboxylases by halophilic investigated strains was performed on Moeller's decarboxylase media with some modifications, based on the formula established by MOELLER (1955). The basal medium used for tests contained (g/L): peptic digest of animal tissue 5, yeast extract 3, dextrose 1, bromocresol purple 0.02, NaCl 100, supplemented with lysine, arginine, or ornithine in percent of 0.5%. 4.5 mL of sterile liquid medium distributed into tubes were inoculated with 0.5 mL liquid bacterial culture. Each inoculated tube was covered with 1 mL sterile mineral oil, and incubated at 35^0C for 24 hours. Fermentation of glucose by bacteria with fermentative metabolism leads to acidification of media, and the pH indicator (bromocresol purple) converts the colour of media from purple to yellow. Tubes were incubated for an additional 24 hours to allow microorganisms to use amino acid. Alkaline conditions created by the enzyme which used amino acid indicated a positive reaction, and were observed when the colour of the medium changed from yellow to purple. If the test organism ferments glucose, but does not produce decarboxylase, the medium remains yellow in colour, and the result is considered negative.

RESULTS AND DISCUSSIONS

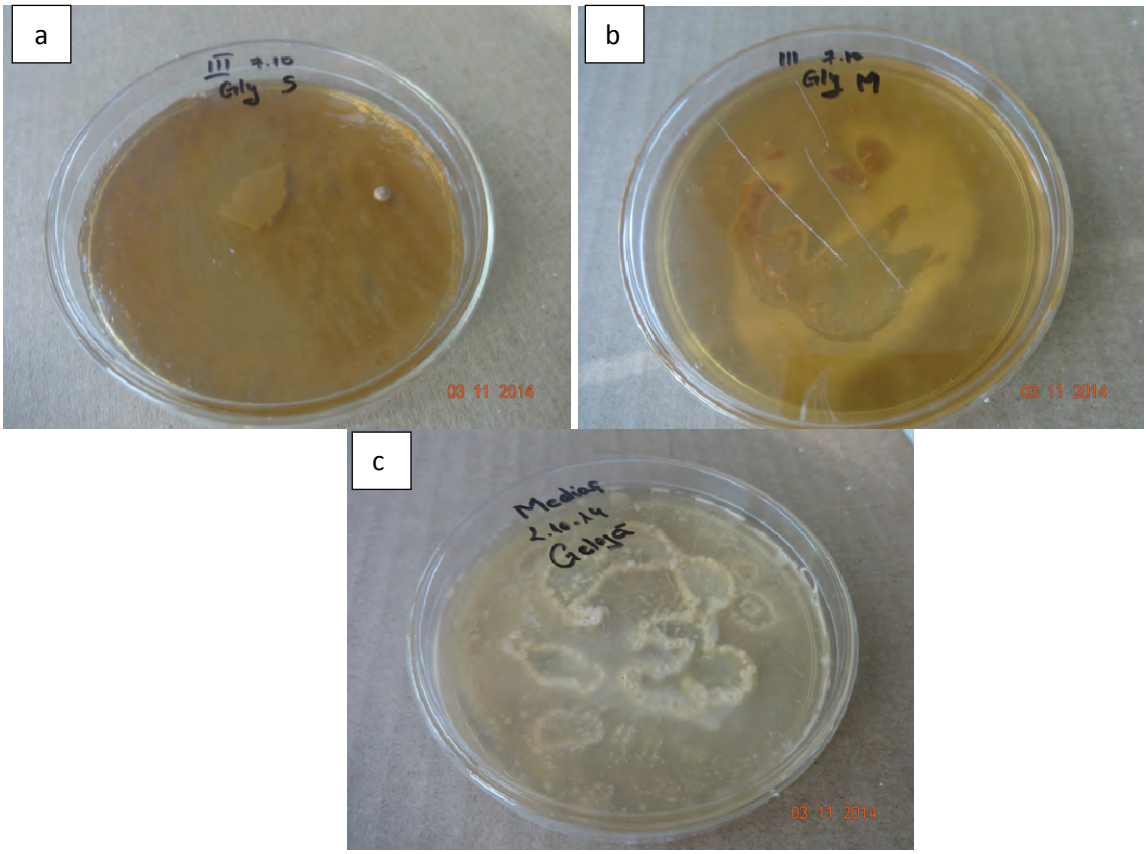
The growth ability of microorganisms on agar media in the presence of waste glycerol

In the first step of the study we try to isolate some putative microorganisms present on the tested types of waste glycerol. In this way it was tested the potential of any type of microorganisms to grow on the agar medium using the waste glycerol as source of microbial strains. The composition of agar medium was changed, using three work variants. The first (I) agar medium variant, contained (g/L): meat extract – 3, peptone – 1, yeast extract – 3, NaCl – 5, agar – 20. The second (II) medium contained (g/L): meat extract – 6, peptone -20, yeast extract – 6, NaCl – 5, agar – 20. The third variant (III) of medium contained (g/L): meat extract – 9, peptone – 20, yeast extract – 9, NaCl – 5, agar – 20. 1 mL of residual glycerol from Mediaș and Slobozia sources was distributed in drops into Petri dishes and the liquefied agar media was poured. After solidification, plates were incubated at 37^0C. In the result, we found that in the third medium variant (III) it was observed the development of two types of bacterial colonies, after 7 days of incubation. The colony which grew on the surface of medium culture was circular, umbonate, opaque, entire, smooth, mauve pigmented with a diameter of 4.7 mm. Also, a large cream colony was observed in depth of the media (Photos 1a, b, c). The bacterial colonies were observed only on variant III of the media with waste glycerol resulted from biodiesel from Slobozia source.

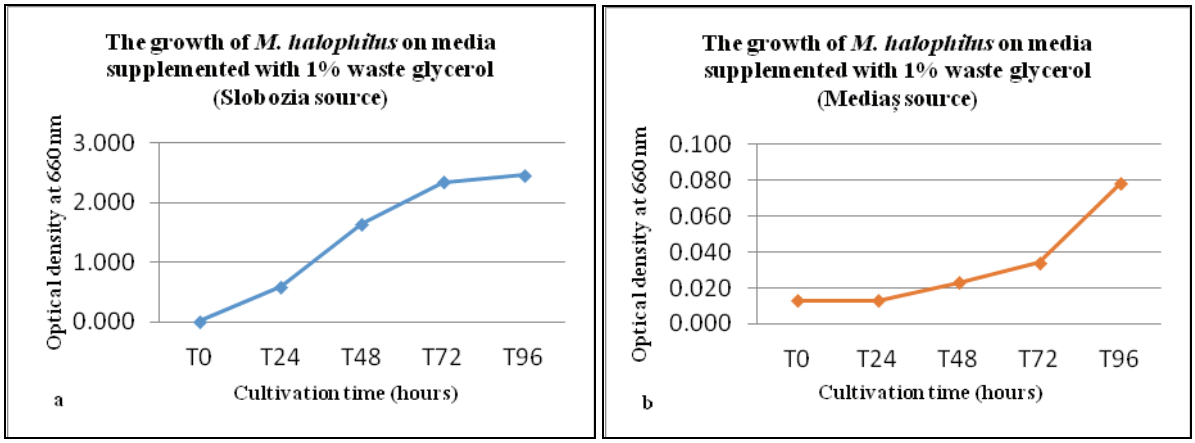
The growth ability of M. halophilus JCM 2472 on R-AGAR media in the presence of 1% waste glycerol

Marinococcus halophilus JCM 2472 strain was selected in our experiments for their ability to produce lipases and decarboxylases. The strain was cultivated on a moderately halophilic (R-AGAR 5% NaCl) medium at 37^0C, pH 7.0-7.2, for 48 hours, and stirring at 130 rpm. Also, it was tested their potential to grow on liquid media with 1% waste glycerol from Mediaș and Slobozia. Growth was monitored by turbidity at OD_{660} using a spectroscopic method (BMG LABTECH FLUOStar Omega – microplate reader) and followed to within 24 hours.

The results revealed that the halophilic strain showed no growth in the presence of residual glycerol from Mediaș (Fig. 1b). On the other hand, the bacterial strain disclosed the ability to grow in the presence of 1% residual glycerol resulted from the biodiesel obtained from Slobozia (Fig. 1a). The differences which appeared in the results may be due to the residual glycerol composition, which consists of some organic matter, phosphate salts and potassium salts, according to the manufacturer data (NEAGU et al., 2015). The residual matters could be a factor which affected the growth of the bacterial strain.



Photos 1a, b, c. (original). The bacterial strains growth on the III variant of agar media, using waste glycerol as inoculum from Slobozia (a), and Mediaș sources (b), and I variant of agar media using waste glycerol from Mediaș (c).



Figures 1a, b. The growth of *M. halophilus* in the presence of 1% waste glycerol from Slobozia (left) and Mediaș (right) sources.

The production of lipases and decarboxylases from halophilic bacterial strains

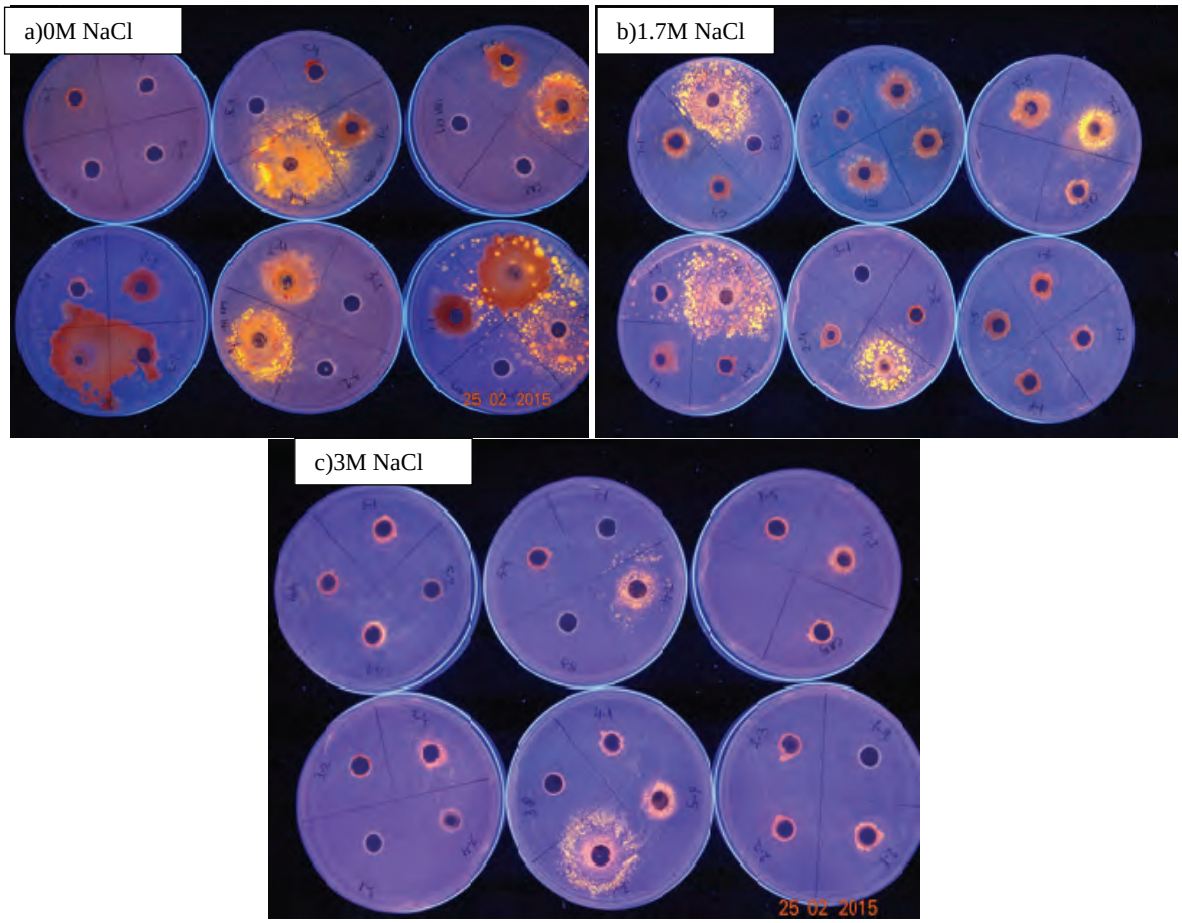
The bacterial strains investigated for their capacity to produce lipases and decarboxylases were isolated from Balta Albă and Techirghiol Lake. The chloride content of the water sample from Balta Albă ranged from 3.5 g/L in a rain period, until to 16 g/L in the absence of rain, with a bacteria density of $3.5x10^5$ colonies forming units per mL (NEAGU et al., 2015). In case of the water sample of Techirghiol Lake, the chloride content was 64 g/L, with $2.1x10^3$

colonies forming units per mL. The number of moderately halophilic bacteria was remarkably higher than that of non-halophilic bacteria. The extreme halophilic bacteria were not recorded. The optimum concentration of NaCl for moderately halophilic bacteria was around 2M NaCl. A number of 76 bacterial strains were isolated from these lakes, but after successive passages on MH media only 30 strains remained cultivable in the laboratory, and used for further experiments. The production of lipases was tested on MH media, as described previously, at different concentration of NaCl (0, 1.7, and 3M). Table 1 shows the lipase and decarboxylase activity of the investigated strains.

Table 1. Lipases and decarboxylases produced by halophilic bacteria isolated from Balta Albă and Techirghiol Lake.

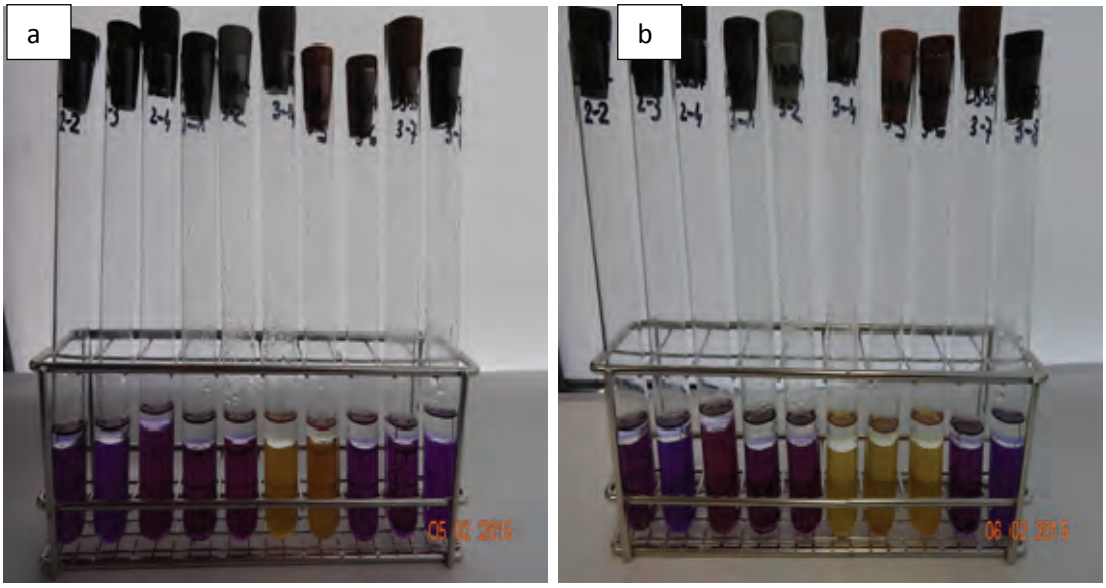
Halophilic strains **Enzyme**	**Gram positive rods**	**Gram negative rods**	**Gram positive cocci**	**Gram negative cocci**
Lipase	0/30	1/30	4/30	3/30
Ornithine-decarboxylase	0/30	0/30	0/30	0/30
Arginine-decarboxylase	0/30	0/30	0/30	0/30
Lysine-decarboxylase	0/30	0/30	0/30	0/30

The results showed that eight halophilic bacterial strains presented in their enzymatic equipment lipases. Also, it was observed the influence of NaCl concentration on lipase activity, which was more intensely at 0M NaCl and decreased with the increasing of NaCl concentration (Photos 2a, b, c). Other strains did not produce lipases, accumulated rhodamine B, formed pink coloured colonies, and did not show orange fluorescence upon UV irradiation.



Photos 2a, b, c (original). The lipase activity of some halophilic bacterial strains tested at 0M (a), 1.7M (b), and 3M(c) NaCl.

The results obtained for the identification of decarboxylase activity tests suggested that the halophilic bacterial strains did not respond positively, even if the acidic conditions were created, and the decarboxylase activity was stimulated, which would have allowed decarboxylation of lysine, arginine, or ornithine amino acids. Figs. 2a, b show the capacity of some investigated strains to ferment dextrose, but the organisms do not produce decarboxylase, and the medium colour remains yellow. Non-utilizers of dextrose did not show any change of the medium colour, which remained purple.



Figures 2a, b. (original). Testing the lysine-decarboxylase activity of some bacterial strains: after 24 hours (a); after 48 hours (b).

CONCLUSIONS

It is already known the potential of halophilic bacteria to produce a variety of extracellular enzymes, and more than that, to tolerate a wide range of salinity, pH, temperature, being valuable organisms for some biotechnological applications (MARGESIN et al., 2001). Some of the investigated bacterial strain isolated from the saline and hypersaline environment from Romania exhibited the ability to synthesize lipases and to use the residual glycerol. Waste glycerol resulted from biodiesel process represents a carbon source that is available at relatively low-cost, and convenient for many applications. Halophilic microorganisms can use glycerol as simple carbon source, and much more than that, to convert it to valuable compounds, such as glycidol, or glycerol carbonate, using an enzymatic cocktail formed by lipases and decarboxylases (NEAGU et al., 2015). Microbial conversion represents an attractive and environmentally friendly alternative for industrial processes.

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