BULK CHEMICALS FROM GLYCEROL – THE BULGARIAN CONTRIBUTION TO BIO-BASED DIOLS PRODUCTION

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Abstract

The syntheses of valuable chemicals from alternative to petroleum sources by the use of microorganisms are unified in the term "white biotechnology". The organic compounds production from waste glycerol is of increased interest, because million tones of this potential substrate are delivered from the biodiesel industry each year. The waste glycerol could be converted by the microbes of genus Klebsiella into several products, as 1,3-propanediol (1,3-PD) and 2,3-butanediol (2,3-BD) are of the major importance.

In the last years, the investigations of bio-based production of 1,3-PD and 2,3-BD were developed in our country. New Klebsiella strains, good producers of both diols, were isolated and identified. The optimal conditions for diols receiving were studied. The nutrient medium compounds optimization was done, and the fermentation requirements for predominant production of different final metabolites were studied.

The potential applications of Bulgarian isolates for glycerol utilization and the economical and environmental impact of this biotechnology were estimated.

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Key words: g*lycerol, 1,3-propanediol, 2,3-butanediol*

1. INTRODUCTION

In the end of XX century a global exhaustion of the natural fuels occurred. The new, environmental concepts of sustainability forced the chemical industries to explore new, bio-based feedstocks for producing bulk chemicals. Alternatives to the petroleum products are the renewable resources as the starch-containing biomass or the waste glycerol, formed by the biodiesel industry.

Despite of the wide applications of glycerol in food, pharmaceutical, cosmetics, and many other industries, it is too costly to refine the crude glycerol to a high purity. When it is used for food, cosmetics, and drugs, further purifications are needed such as bleaching, deodoring, and ion exchange to remove trace substances. The glycerol is generally treated and refined through filtration, chemical additions, and fractional vacuum distillation to yield various commercial grades. Purifying it to that stage, however, is costly and generally out of the range of economic feasibility for the small to medium sized plants. Much more appropriate would be its direct use for biosynthesis of valuable

products during biotechnological process. The effective usage of crude glycerol to specific products would seriously cut down the biodiesel production costs.

The purpose of this review is to display the main fermentative products of the microbial glycerol conversion, the available strains – producers of important metabolites and to trace the prospects for their industrial scale production. It aims also to clarify the perspectives for diols production development in our country, to estimate the potential of Bulgarian *K. pneumoniae* isolates and to evaluate the economical and environmental impact of this "white" biotechnology.

2. THE CRUDE GLYCEROL FROM BIODIESEL PRODUCTION

Biodiesel is a renewable fuel produced from vegetable oils such as rape seed oil, sunflower seed oil, soybean oil and also used frying oils or animal fats. Its applications are found in food, drug, cosmetic and tobacco industries. In the transport sector, it may be effectively used both when blended with fossil diesel fuel and in pure form. Biodiesel has been demonstrated to have significant environmental benefits in terms of decreased global warming impacts**,** reduced emissions**,** greater energy independence and a positive impact on agriculture. The principal by–product of biodiesel manufacturing is the crude glycerol, which is about 10 % of the weight of the used vegetable oil (Dasari *et al.*, 2005). Biodiesel production in Europe and in the United States also increased dramatically in the past few years. The United States has increased its production from two million gallons in 2000 to 250 million gallons in 2006 and an estimated of 650 million gallons in 2010. According to the National Biodiesel Board, there are 105 plants in operation as of early 2009 with an annual production capacity of 864 million gallons. A rapid expansion in biodiesel production capacity is being observed not only in developed countries but also in developing countries such as China, Brazil, Argentina, Indonesia, and Malaysia. The global biodiesel market is estimated to reach 37 billion gallons by 2016, growing at an average annual growth of 42%. Europe will continue as the major Biodiesel market for the next decade or so, closely followed by the US market (Li *et al*., 2008).

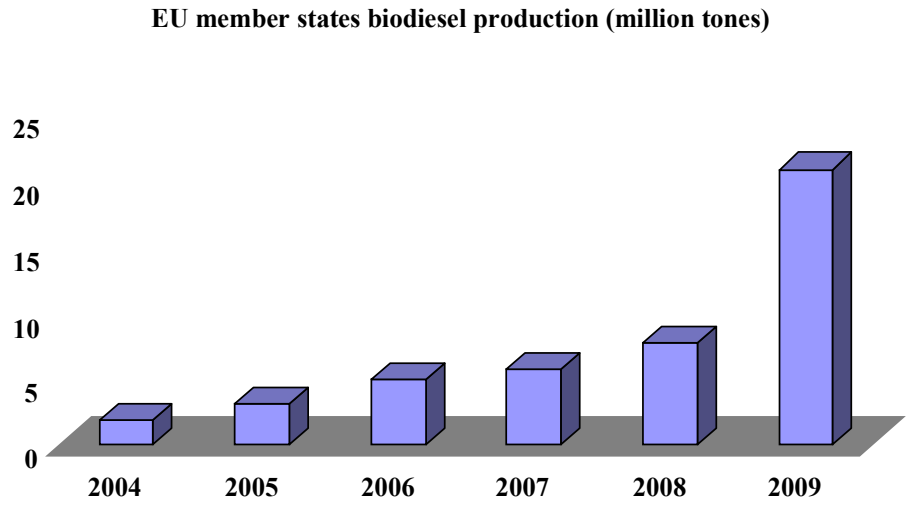


Fig. 1 The statistics of the European biodiesel board, revealing the increase in biodiesel production in Europe during last years

For a current biodiesel production in Europe, from 20,9 million tones of biodiesel, obtained in 2009, about 2 million tones waste glycerol have been formed. As the biodiesel manufacturing is a constantly growing sector (Fig. 1), the waste glycerol turns in inexhaustible source for biotechnological processes.

3. VALUABLE CHEMICALS, OBTAINED FROM GLYCEROL AND THEIR INDUSTRIAL APPLICATIONS

Among the promising bulk chemicals, 1,3-propanediol (1,3-PD) and 2,3-butanediol (2,3-BD) are two bio-based diols which have a wide range of applications in cosmetics, foods, transport fuels (antifreezes, lubricants, or fuel additives), and medicines as well as in the production of polymers (Xiu and Zeng, 2008).

3.1. 1,3-Propanediol

Because of its special properties, the application of 1,3-PD is mainly in the production of polymers, such as polyesters, polyethers, and polyurethanes. A 1,3-PD-based new polyester, poly(trimethylene terephthalate) (PTT) has received much attention because of several unique properties for the production of fibers (Kurian, 2005). So far 1,3-PD is mainly manufactured by chemical synthesis, which requires expensive catalyzers, high temperature, high pressure, and high level of safety measurement. Considering the yield and recovery of product, environmental protection, and sustainable development of 1,3-PD, much attention has been paid to its microbial production, based on glycerol (Xiu and Zeng, 2008).

3.2. 2,3-Butanediol

2,3-BD has large number of industrial applications as a reagent in various chemical syntheses. The dehydration of 2,3-BD yields the industrial solvent methyl ethyl ketone. Further dehydration produces 1,3- butadiene, which is the building block of synthetic rubber (van Haveren *et al.*, 2007). And the high octane rating of 2,3-BD makes it a potential aviation fuel (Wu *et al.*, 2008). Besides, 2,3-BD has potential applications in the manufacture of printing inks, perfumes, fumigants, moistening and softening agents, explosives, plasticizers, foods and pharmaceuticals (Syu, 2001). It can also be easily dehydrogenated into acetoin and diacetyl which are flavoring agents used in dairy products, margarines, and cosmetics (Garg and Jain 1995).

3.3. The best diol producers and the biochemical pathways of 1,3-PD and 2,3-BD synthesis

Table 1. Bacterial strains, converting glycerol into valuable chemicals

Species	Strain	Main product	Reference
K. oxytoca	Lin	1,3-PD	Homann *et al*., 1990
	M5al	1,3-PD	Yang *et al*., 2007
K. pneumoniae	Bs2e	1,3-PD	Homann *et al.*, 1990
	KG1	1,3-PD	Zhao *et al.*, 2008
	DSM 2026	1,3-PD	Biebl *et al*., 1998; Mu *et al.*, 2006; Chen *et al.*, 2003; Zhang *et al.*, 2008
	M5aL (M5al)	1,3-PD	Cheng *et al*., 2004; Du *et al*., 2007
	ME-308	1,3-PD	Ji *et al*., 2009
	AC15	1,3-PD	Zheng *et al*., 2008
	ECL 2103	1,3-PD	Jonhson and Lin, 1987

	TUAC 01		Hao *et al*., 2008a,b
	XJPD-Li	1,3-PD	Ma *et al*., 2009
	G9, G31, G46	1,3-PD	Petrova *et al*., 2009;
	G31	2,3-BD	Petrov and Petrova, 2009 a,b
K. planticola	DSM 3060	Ethanol	Homann *et al*., 1990
K. terrigena	DSM 2687	Ethanol	Homann *et al*., 1990
C. freundii	DSM 30040		Homann *et al*., 1990;
	T2, T3	1,3-PD	Boenigh *et al.*, 1993;
	Zu, Go		Biebl and Marten, 1995
Cl. butyricum	SH1 (DSM 5431)		Forsberg, 1987
	AK1 (DSM 5430)	1,3-PD	Gunzel *et al.*, 1991
	DSM 523,	Butanol	Biebl, 1991
	W3α,	Acetic acid	Biebl *et al.*, 1992
	ATCC 11914		Biebl and Marten, 1995
Cl. kainatoi	DSM 523	1,3-PD	Biebl *et al.*, 1992
E. aerogenes	HU-101		
	DSM 30053	Ethanol	Ito *et al.*, 2005; Homann *et al.*, 1990

The glycerol is usually metabolized during mixed-acid fermentation, gaining more than five liquid fermentative products, including 1,3-propanediol, acetic, succinic and lactic acids, ethanol, 2,3-butanediol, CO_2, H_2, etc. The strains, capable to convert glycerol are representatives of the genera *Klebsiella*, *Clostridium*, *Citrobacter*, *Enterobacter* and *Lactobacillus*. The strains, capable to produce high amounts of valuable chemicals from glycerol and their species affiliation are listed in Table 1.

In *Klebsiella*, *Citrobacter*, *Clostridium* and *Enterobacter*, glycerol is metabolized both oxidatively and reductively. In the oxidative pathway (Fig. 2), the NAD^+-dependent enzyme *glycerol dehydrogenase* catalyzes the conversion of glycerol to dihydroxyacetone and the glycolytic enzyme *dihydroxyacetone kinase* phosphorylates the latter product, which is then funneled to glycolysis.

The reducing pathway is catalyzed by coenzyme B_{12}-dependent *glycerol dehydratase* and related *diol dehydratases* converting glycerol to 3-hydroxypropionaldehyde and by the $NADH+H^+$-dependent enzyme *1,3-propanediol dehydrogenase* (1,3-propanediol-oxydoreductase, reducing 3-hydroxypropionaldehyde to 1,3-propanediol and regenerating NAD^+. The final 1,3-propanediol is highly specific for glycerol fermentation and cannot be obtained from any other anaerobic conversion (Da Silva *et al.*, 2009). The establishment of optimal conditions for 2,3-BD synthesis must include enhancement of the oxidative pathway of the bioconversion of glycerol and suppression of the reductive branch. Through the oxidative pathway glycerol is dehydrogenated; phosphorylated and directed towards the glycolysis, producing variety of final metabolites, including 2,3-butanediol.

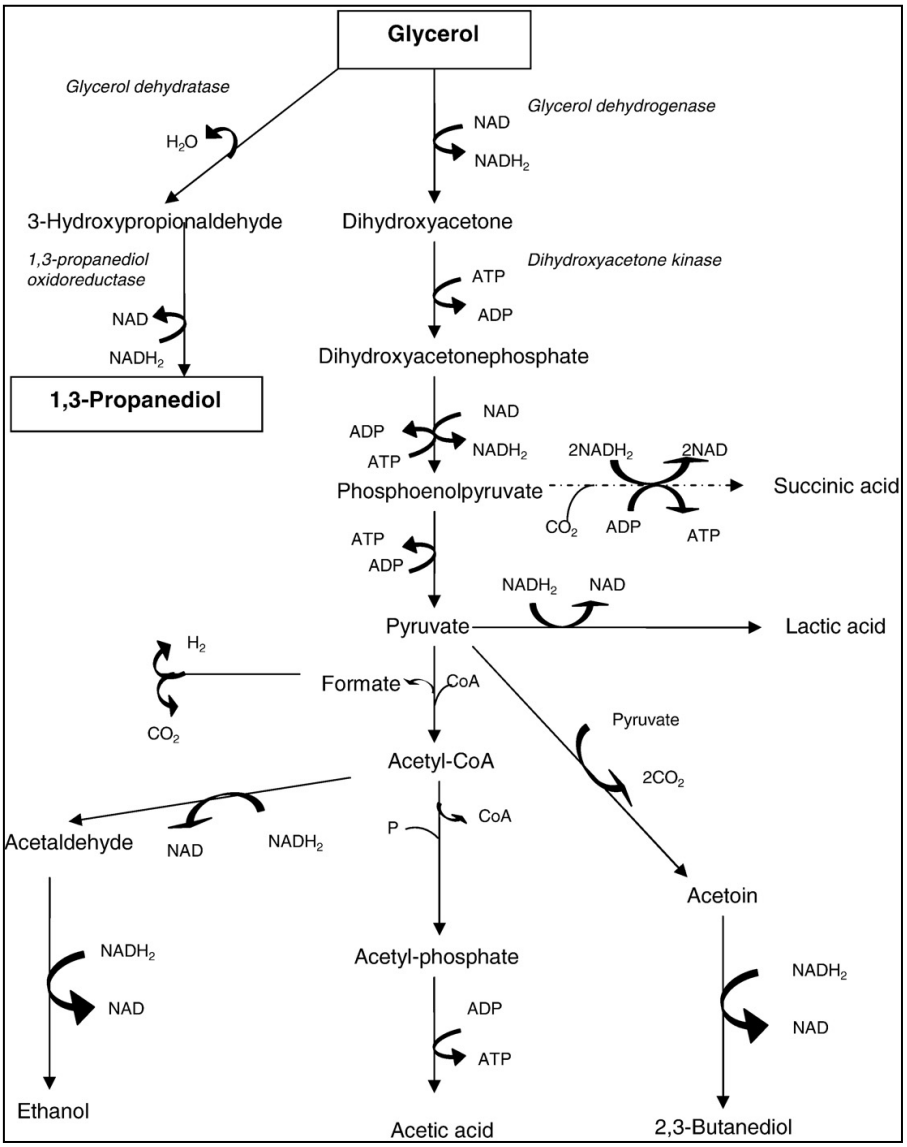


Fig. 2 The possible end products of the glycerol fermentation by microorganisms (Saxena *et al.*, 2009)

This branch provides energy and reducing equivalents (NADH) for the biosyntheses. The most energy-advantageous end product of this pathway for the bacteria, would be acetic acid, as its formation is connected with NAD^+ regeneration, coenzyme A recycling and also with more chemical energy profit, compared to all other final products, such as lactic and succinic acids, ethanol and 2,3-BD (Syu, 2001). On the other hand, the overdone secretion of acetic acid leads to the cell growth inhibition by the accumulation of its undissociated form (Biebl *et al.* 1998).

4. OPTIMAL FERMENTATION CONDITIONS FOR DIOL PRODUCTION BY *K. PNEUMONIAE* STRAINS

4.1. Strain specificity

Klebsiella pneumoniae ferments glycerol to both diols in a high yield and productivity. Considering the effectiveness of the process and fermentation requirements, *Klebsiella* is highly productive and in the same time, quite unpretentious in comparison with other genera, producing diols. There have been isolated a number of *Klebsiella* strains, able to ferment glycerol, belonging to species *K. pneumoniae, K. oxytoca* and *K. planticola.*

4.1. Substrate concentration

Chen *et al.* (2004), examining the formation of key enzymes, involved in glycerol consumption, affirmed that the optimal substrate concentration is 30 g/l. Others marked, that 1,3-PD productivity of ATCC 25855 seriously dropped when 60 g/l substrate was used and was totally inhibited by 100 g/l glycerol (Huang *et al.*, 2002). In these terms, the concentration of 50 g/l glycerol, which could be completely consumed by G31 and G46, indicates that the enzymes, involved in the glycerol utilization by both strains, possessed relatively strong resistance to substrate inhibition.

4.2. Media composition

Several nutrient media were developed in order to produce higher amounts of diols by *Klebsiella*. All of them contained a mix of phosphates, yeast extract and trace elements solution. Best productivity of 1,3-propanediol was obtained in the medium of Zhao *et al*. (2009), but for enhanced production of 2,3-butanediol the salts, containing $Co^{2}+$ ions had to be excluded (Petrov and Petrova, 2009b).

4.3. Aeration

Although the process of glycerol utilization by *Klebsiella* was considered as anaerobic for many years, some authors developed different aeration strategies aiming to improve the fermentation (Chen *et al*., 2003; Cheng *et al.*, 2004) or performed it aerobically (Hao *et al.*, 2008a). Statistical optimization via central composite design was done by Zheng *et al.* (2008) revealed that aeration, beneficial for 1,3-PD production is 0.6 vvm.

4.4. pH

Totally different amounts of diol products could be obtained in dependence of the pH during the cultivation The comparative study of the fermentation products, obtained without pH control and those at pH, maintained at pH 7 by many authors showed that the pH is a powerful trigger in alteration of the process to the aimed product 1,3-PD or 2,3-BD. At uncontrolled pH, the majority of the strains synthesize mix of diols, in which the share of 1,3-PD and 2,3-BD vary from 30 to 50% (Biebl *et al*., 1998, 1999). As 2,3-BD is highly valuable product too, when the goal is the receiving of both diols almost without by-products, the fermentation could be carried out without any pH control. At these conditions, the most successive conversion of glycerol into diols could be completed by strains, producing the lower amounts of acetate and lactate. The pH maintenance at the neutral point definitely directed the process to 1,3-PD production and its concentration increase, up to thrice in comparison with the non-controlled pH process (Petrov and Petrova, 2009a). However, this increases the share of lactic acid and ethanol too.

Summarizing all factors, influencing the glycerol fermentation, two of them must be noticed as most important: the strain specificity and the pH. Not crucial, but also substantial, were the media composition and the aeration. The highest amounts of 1,3-PD, obtained until now, are reported during aerobic fed-batch by the use of strains *K. pneumoniae* XJPD-Li (65.26 g/l, Ma *et al*., 2009) and

TUAC01 (70.6 g/l, Hao *et al*., 2008b). The most successful process for 2,3-BD receiving was the fed-batch with *K. pneumoniae* G31 (Petrov and Petrova, 2009b).

The Bulgarian contribution to the improvement of diols production from glycerol covers new strains isolation and fermentation optimizations. Three Bulgarian *K. pneumoniae* strains (G9, G31 and G46) were isolated and identified (Table 1) and were evaluated as perspective for diols production. The conditions, favorable for either 1,3-PD or 2,3-BD were investigated. The optimization of the media content and cultivation conditions, aiming predominant 2,3-BD synthesis during fed-batch fermentations was done. It was revealed that 2,3-BD was formed in unequal amounts in course of a complicated pH self-control mechanism (Petrov and Petrova, 2009b). All experiments were done with pure glycerol as a substrate. The future investigations will be performed by the use of waste industrial glycerol from the biodiesel preparations.

In conclusion, the utilization of the abundant and low-priced glycerol as a substrate for production of high-value products represents a promising route to achieve economic viability in the biofuels industry. If fuels and reduced chemicals are targeted, there are many advantages for the use of glycerol over sugars (Yazdani and Gonzalez, 2007) for diminishing the capital and operational costs.

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