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Research review paper

Glycerol: A promising and abundant carbon source for industrial microbiology

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ABSTRACT

Petroleum is the main energy source utilized in the world, but its availability is limited and the search for new renewable energy sources is of major interest. Biofuels, such as ethanol and biodiesel, are among the most promising sources for the substitution of fossil fuels. Biodiesel can replace petroleum diesel, as it is produced from animal fats and vegetable oils, which generate about 10% (w/w) glycerol as the main by-product. The excess glycerol generated may become an environmental problem, since it cannot be disposed of in the environment. One of the possible applications is its use as carbon and energy source for microbial growth in industrial microbiology. Glycerol bioconversion in valuable chemicals, such as 1,3-propanediol, dihydroxyacetone, ethanol, succinate etc. is discussed in this review article.

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1. Introduction

The demand for biofuels is currently on the rise worldwide and the use of biomass is one of the most promising alternatives. Brazil was a

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pioneer in the use of clean (with respect to CO_2 balances) energy sources (biofuel) when its government decided to replace gasoline with alcohol fuel in 1973. In the mid 1980s, about 95% of the automobiles produced in Brazil were changed to ethanol combustion. Flex fuel vehicles, which were engineered in 2003, account for more than 80% of the automobiles sold in Brazil. The use of biofuels will certainly continue to grow, as the availability of petroleum is clearly limited. Estimations point to a gradual decline in petroleum production starting in 2010 and, according to some projections, the reserves are thought to become completely depleted by 2050 (Campbell and Laherrère, 1998).

Another biofuel originated from biomass is biodiesel, produced from vegetable oils and animal fats. Just as ethanol can replace gasoline in Otto cycle motors, biodiesel can replace the diesel in diesel engine motors. At the present time, the European Union is the principal biodiesel producer, with 82% of biodiesel produced in the world in 2003 (Demirbas and Balat, 2006). According to the European Biodiesel Board (EBB, 2006), the estimated production of biodiesel in 2005 was about 3.2 million tons and the production capacity in 2006 was estimated to be about 6069 million tons. The USA is the second major world producer and produced about 250,000 tons of biodiesel in 2005 (EBB, 2006).

Biodiesel has been mainly used in Germany, which is currently the largest producer and consumer of this biofuel, exceeding 2.5 billion liters annually. A number of other countries have recently adopted the use of biodiesel, usually as an admixture to petroleum diesel in different proportions. In Brazil, the addition of 2% biodiesel (B2) is obligatory since January of 2008, increasing to 5% (B5) in 2013. Brazil will become a major producer and consumer of biodiesel for two reasons: first, the use of alcohol to fuel cars has a long tradition in the Brazilian culture and, secondly, the conditions for cultivating oleaginous plants are extremely favorable in many areas. Furthermore, the agricultural know-how to grow these plants is available and also large areas of cultivatable land have not yet been explored.

Simple alcohol glycerol (1,2,3-propanetriol, also glycerin or glycerine) is the principal by-product obtained during transesterification of vegetable oils and animal fats (Solomon et al., 1995; Barbirato et al., 1997a,b, 1998; Colin et al., 2001). Glycerol is abundant in nature, since it is the structural component of many lipids. It is also one of the principal compatible solutes, being widely produced in response to decreased extracellular water activity during osmoregulation in yeasts (Wang et al., 2001). Due to its ample occurrence in nature, many known microorganisms can naturally utilize glycerol as a sole carbon and energy source. These microorganisms have attracted attention to the potential use in bioconversion of abundant glycerol produced from biodiesel (Solomon et al., 1995; Barbirato and Bories, 1997; Menzel et al., 1997a). Glycerol may substitute traditional carbohydrates, such as sucrose, glucose and starch, in some industrial fermentation processes.

Glycerol bioconversion adds significant value to the productivity of the biodiesel industry. Throughout this paper, examples of possible biotechnological production processes based on glycerol demonstrate that this simple chemical is a promising abundant new carbon source for industrial microbiology.

2. Glycerol and biodiesel

Glycerol can be produced either by microbial fermentation or chemical synthesis from petrochemical feedstock. It can also be recovered from soap manufacturing. In the traditional process of the latter, glycerol is released as a by-product during the hydrolysis of fats. This process is currently of less importance, since soap has been largely replaced by detergents (Wang et al., 2001).

Biodiesel is produced from vegetable oils and animal fats through transesterification with, for instance, ethanol or methanol (alcoholysis), generally catalyzed by NaOH or KOH (Fig. 1) and glycerol represents 10% (v/v) of the ester (Papanikolaou et al., 2002; González-Pajuelo et al., 2004; Mu et al., 2006). Europe mainly uses rapeseed oil for biodiesel production. In Brazil, oils from soybean, sunflower, African oil palm (*Elaeis guineensis*), castor oil and *Jatropha curcas* are used.

In some European countries, the production of glycerol has increased significantly due to biodiesel uptake. As a consequence, prices have fallen and the majority of companies that chemically produced glycerol have shut down business (Dharmadi et al., 2006; Deckwer, 1995). In the EU, some biodiesel companies have severe problems getting rid of excess glycerol and disposal is quite expensive. The collapse of glycerol prices causes major problems to these companies (Willke and Vorlop, 2004; Dharmadi et al., 2006).

It is reported that approximately 75–95% of the final costs of biodiesel arise from the cost of raw material (Yuste and Dorado, 2006). Biodiesel is now widely accepted as a renewable fuel and a major goal for the future will be to reduce the costs related to biodiesel production. One possibility is to use inexpensive or low-cost raw materials, such as vegetable oils used for frying (Yuste and Dorado, 2006). The reduction in taxes for biofuels is another important approach. Considering the increasing need for renewable fuels throughout the world and the increasing demand and production of biodiesel, an excess of glycerol will be available in the world. Since glycerol can be used as a carbon source in industrial microbiology, this by-product adds value to the productive chain of the biodiesel industry, contributing to their competitiveness.

Glycerol is present in many applications in the cosmetic, paint, automotive, food, tobacco, pharmaceutical, pulp and paper, leather and textile industries. It also is used as a feedstock for the production of various chemicals (Wang et al., 2001). New applications are being evaluated in the food industry, the polyglycerol and polyurethane industry, the field of wood stabilizers and production of small molecules, such as dihydroxyacetone, glyceric and hydroxypyruvic acids and glycerol carbonate (Claude, 1999). Glycerol has also been considered as a feedstock for new industrial fermentations in the future (Wang et al., 2001). Thus, one of the many promising applications for the use of glycerol is its bioconversion to high value compounds through microbial fermentation. Glycerol is not only cheap and abundant, but its greater degree of reduction than sugars offers the opportunity to obtain reduced chemicals, as succinate, ethanol, xylitol, propionate, hydrogen, etc. at higher yields than those obtained using sugars (Dharmadi et al., 2006).

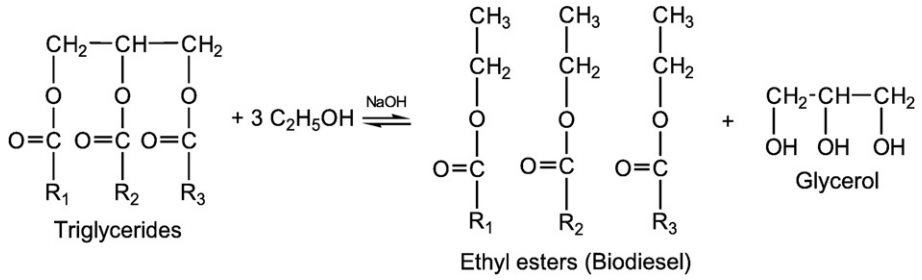

Fig. 1. Transesterification reaction of a triglyceride with ethanol (alcoholysis), utilizing NaOH as a catalyst and rendering biodiesel (mixture of fatty acid ethyl esters) and glycerol.

3. Microbial growth with glycerol as carbon and energy source

3.1. Glycerol uptake

Like other small uncharged molecules, glycerol can cross the cytoplasmic membrane through passive diffusion. However, cells limited to passive uptake have a growth disadvantage at low concentrations of substrate. Glycerol uptake is frequently cited as the only example of transport mediated by facilitated diffusion across the *Escherichia coli* inner membrane (Voegele et al., 1993). Facilitated diffusion is achieved by an integral membrane protein, the glycerol facilitator GlpF (Heller et al., 1980; Voegele et al., 1993; Darbon et al., 1999). Intracellular glycerol is subsequently converted to glycerol-3-phosphate by glycerol kinase (GlpK). Glycerol-3-phosphate remains trapped in the cell until it is further metabolized because it is not a substrate for the glycerol facilitator (Voegele et al., 1993; Darbon et al., 1999; Braun et al., 2000).

GlpF acts as a highly selective channel, also conducting poly-alcohols and urea derivatives, for which it is stereo-selective and enantio-selective (Braun et al., 2000; Fu et al., 2000). All these channels are strictly selective for non-ionic compounds, including hydroxide and hydronium ions, thus preventing the dissipation of the membrane potential (Braun et al., 2000; Fu et al., 2000). The influx of glycerol mediated by GlpF is 100- to 1000-fold greater than expected for a transporter and is non-saturable at a glycerol concentration of >200 mM (Fu et al., 2000).

In *Saccharomyces cerevisiae*, the flow of glycerol across the plasma membrane is controlled either by passive diffusion, a channel protein or an active uptake mechanism (Wang et al., 2001).

3.2. Microbial metabolism of glycerol

A number of microorganisms are able to grow anaerobically on glycerol as the sole carbon and energy source, such as *Citrobacter freundii* (Homann et al., 1990; Daniel et al., 1995; Seifert et al., 2001), *Klebsiella pneumoniae* (Forage and Foster, 1982; Tong et al., 1991; Menzel et al., 1997b; Biebl et al., 1998; Németh et al., 2003), *Clostridium pasteurianum* (Luers et al., 1997; Macis et al., 1998; Biebl, 2001), *Clostridium butyricum* (Abbad-Andaloussi et al., 1995; Biebl, 1991; Biebl et al., 1992; Himmi et al., 1999; Malaoui and Marczak, 2001; Colin et al., 2001), *Enterobacter agglomerans* (Barbirato et al., 1996; Barbirato and Bories, 1997; Barbirato et al., 1997a), *Enterobacter aerogenes* (Ito et al., 2005) and *Lactobacillus reuteri* (Talarico et al., 1988, 1990).

In *Klebsiella*, *Citrobacter*, *Clostridium* and *Enterobacter*, glycerol is metabolized both oxidatively and reductively (Zhu et al., 2002). In the oxidative pathway, the NAD^+-dependent enzyme *glycerol dehydrogenase* (EC 1.1.1.6) catalyzes the conversion of glycerol to dihydroxyacetone and the glycolytic enzyme *dihydroxyacetone kinase* (EC 2.7.1.29) phosphorylates the latter product (Daniel et al., 1995; Luers et al., 1997; Macis et al., 1998), which is then funneled to glycolysis. The reducing pathway is catalyzed by coenzyme B_{12}-dependent *glycerol dehydratase* (EC 4.2.1.30) and related *diol dehydratases* (EC 4.2.1.28) (Toraya et al., 1978; Forage and Foster, 1982; Knietsch et al., 2003), converting glycerol to 3-hydroxypropionaldehyde (Toraya et al., 1980; Tong et al., 1991; Seifert et al., 2001), and by the $NADH+H^+$-dependent enzyme *1,3-propanediol dehydrogenase* (1,3-propanediol-oxydoreductase, EC 1.1.1.202), reducing 3-hydroxypropionaldehyde to 1,3-propanediol and regenerating NAD^+ (Macis et al., 1998; Skraly et al., 1998; Ahrens et al., 1998; Veiga da Cunha and Foster, 1992; Németh et al., 2003) (Fig. 2). The final 1,3-propanediol

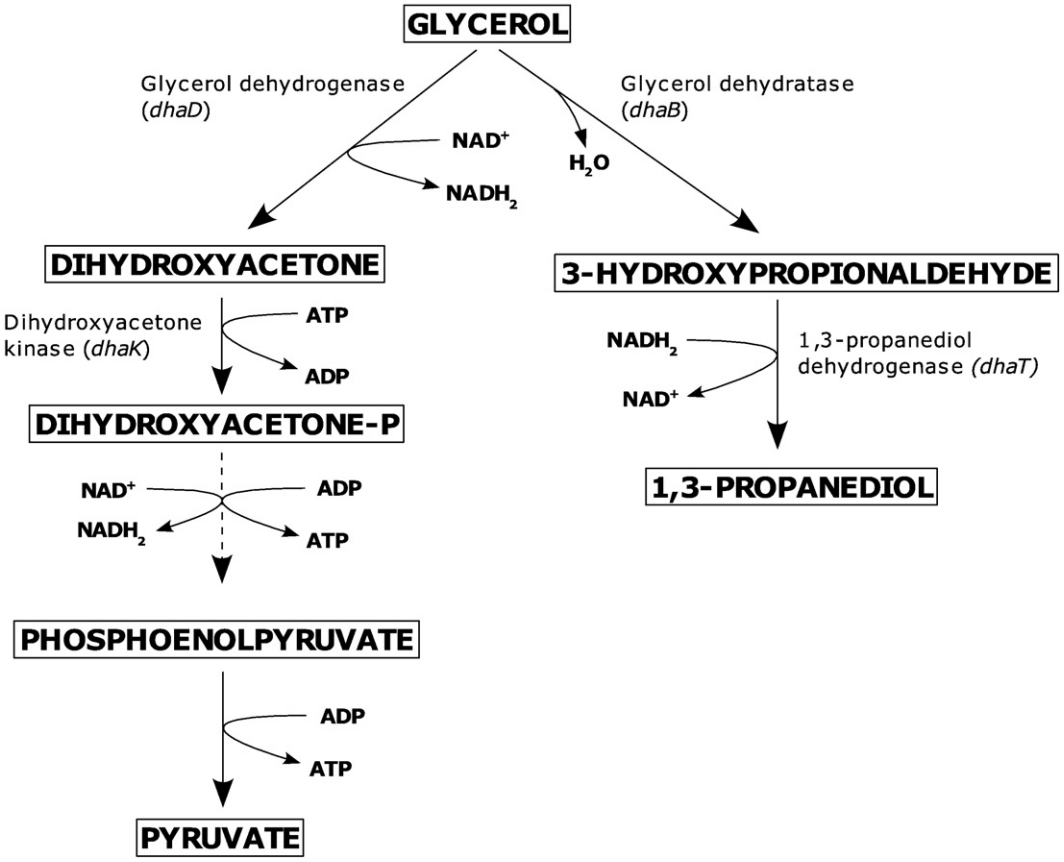

Fig. 2. Fermentative patterns of glycerol dissimilation dependent on 1,3-PDO formation. The key enzymes of the *dha* regulon and respective genes related to glycerol metabolism are shown. Pyruvate will be reduced to different organic compounds dependent on microorganism and fermentation conditions, regenerating NAD^+ (adapted from Bouvet et al., 1995; Barbirato et al., 1997b; Menzel et al., 1997a; Biebl, 2001).

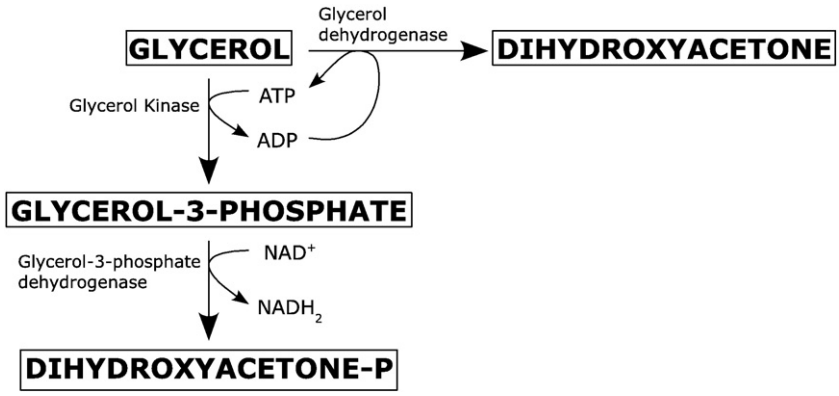

Fig. 3. Pathways for growth and DHA production by *G. oxydans* in glycerol. The membrane-bound glycerol dehydrogenase leads to an extracellular production of DHA and uses O_2 as the final acceptor of electrons and reduced equivalents by means of ubiquinone and cytochrome *o*. DHA-P is catabolized by means of the pentose-phosphate pathway (adapted from Bories et al., 1991; Claret et al., 1994).

(1,3-PDO) product is highly specific for glycerol fermentation and cannot be obtained from any other anaerobic conversion (Homann et al., 1990; Deckwer, 1995).

In *K. pneumoniae* (Forage and Lin, 1982) and *C. freundii*, the genes encoding the functionally linked activities of glycerol dehydratase (*dhaB*), 1,3-PDO dehydrogenase (*dhaT*), glycerol dehydrogenase (*dhaD*), and dihydroxyacetone kinase (*dhaK*) are encompassed by the *dha* regulon (Zhu et al., 2002) (Fig. 2). The 1,3-PDO operon of *C. butyricum* is composed of three genes, a different type of glycerol dehydratase (*dhaB1*), its activator protein (*dhaB2*) and *dhaT* (Raynaud et al., 2003). In this bacterium, glycerol dehydratase is extremely oxygen sensitive, strongly associated with the cell membrane and vitamin-B_{12} independent (Saint-Amans et al., 2001; Raynaud et al., 2003; González-Pajuelo et al., 2004, 2005a,b, 2006).

In *S. cerevisiae* and a number of other yeasts, glycerol is degraded via dihydroxyacetone or via glycerol-3-phosphate (Wang et al., 2001). In the latter, glycerol is converted to glycerol-3-phosphate through *glycerol kinase* (EC 2.7.1.30), which can be used either as a precursor for lipid biosynthesis or conversion to dihydroxyacetone phosphate and can then either be transformed to glyceraldeyde-3-phosphate by *triose phosphate isomerase* (EC 5.3.1.1) in glycolysis or can serve as a substrate for the synthesis of other metabolites (Wang et al., 2001). Similar pathways for glycerol oxidation (*glp* regulon) are present in *K. pneumoniae* (Ruch et al., 1974; Forage and Lin, 1982), *Gluconobacter oxydans* (Bories et al., 1991; Claret et al., 1994) and *C. acetobutylicum* (González-Pajuelo et al., 2006) (Fig. 3). According Ruch et al. (1974), the glycerol-3-phosphate pathway is responsible for the aerobic degradation of glycerol in *K. pneumoniae* (formerly *K. aerogenes*), while the dihydroxyacetone pathway is responsible for the anaerobic degradation of this substrate.

Fermentation from glycerol to ethanol or butanol by *C. pasteurianum* does not depend on the formation of by-products (Biebl, 2001), since hydrogen carriers are completely regenerated in the pathway (Biebl et al., 1998). Another example of a redox-balanced process is the conversion of glycerol into succinic acid. Although the pathways for ethanol and succinate are equivalent regarding the overall redox balance, the energetic contribution of the ethanologenic pathway is much higher, as 1 ATP is produced per each molecule of glycerol converted into ethanol, while production of energy in the succinate pathway is limited to the potential generation of a proton motive force by fumarate reductase (Dharmadi et al., 2006) (Fig. 4).

4. Glycerol bioconversion in industrial microbiology

4.1. 1,3-propanediol

The diglycol 1,3-propanediol (Trimethylene glycol, propylene glycol) is the principal product obtained through glycerol fermentation. 1,3-PDO was first observed as a product of the fermentation of glycerol in 1881 (Werkman and Gillen, 1932). It is one of the oldest known fermentation products, but little attention was paid to this microbial pathway for over a century (Biebl et al., 1999). 1,3-PDO presents several interesting applications (Tong et al., 1991; Deckwer, 1995; Colin et al., 2000; Cheng et al., 2004; Lin et al., 2005). It can be used as a monomer for polycondensations to produce plastics with special properties, i.e. polyesters, polyethers and polyurethanes (Deckwer, 1995; Colin et al., 2001; Wilke, 1999; Himmi et al., 1999; Yang et al., 2007; Du et al., 2006), as a monomer for cyclic compounds (Knietsch et al., 2003), as a polyglycol-type lubricant and it also may serve as a solvent (Papanikolaou et al., 2000).

1,3-PDO is an emerging commodity chemical (Cameron et al., 1998) and renewed interest in its biotechnological production has recently arisen (Abbad-Andaloussi et al., 1995; Zhang et al., 2006b). The development of a new polyester, polypropylene terephthalate (PPT or polytrimethylene terephthalate — PTT) from 1,3-propanediol requires a drastic increase in the production of this chemical (Raynaud et al., 2003; González-Pajuelo et al., 2005b, 2006). PPT is a biodegradable polyester that has great potential for use in carpet and textile manufacturing and is related to polyethylene terephthalate (PET) and polybutylene terephthalate (PBT) (Hao et al., 2006). The chemical companies Shell and DuPont hold patents related to new commercial PPT polymers obtained from 1,3-propanediol. Shell has developed Corterra® and DuPont has developed Sorona® and Hytrel®.

PPT was developed and patented in 1941, but application was limited since the precursor 1,3-PDO was too expensive. Thus, many companies tried to develop new and more efficient technologies for 1,3-PDO production. In the new Shell process, 1,3-PDO is chemically obtained from the reaction of ethylene oxide with carbon monoxide and hydrogen. In the DuPont development, 1,3-PDO is produced fermentatively from glucose by recombinant microorganisms (Biebl et al., 1999; Chotani et al., 2000; Nakamura and Whited, 2003). According to Nakamura and Whited (2003), the comparison between glucose and glycerol considering substrate and yield has favored the direct D-glucose route, mainly because glucose was cheaper. However, with the abundant surplus of glycerol from biodiesel production, the glucose route is probably more expensive now and glucose may be used for other applications, such as alcohol fermentation. Furthermore, glucose competes directly with food and feed production, which is not the case for glycerol.

Over 10^5 tons of 1,3-PDO are produced yearly, mostly through chemical synthesis (Németh et al., 2003; Zhang et al., 2006a). 1,3-PDO is chemically produced through two different routes: hydration of acrolein and hydroformylation of ethylene oxide (Cameron et al., 1998; Hao et al., 2006; Yang et al., 2007). Chemical synthesis requires high

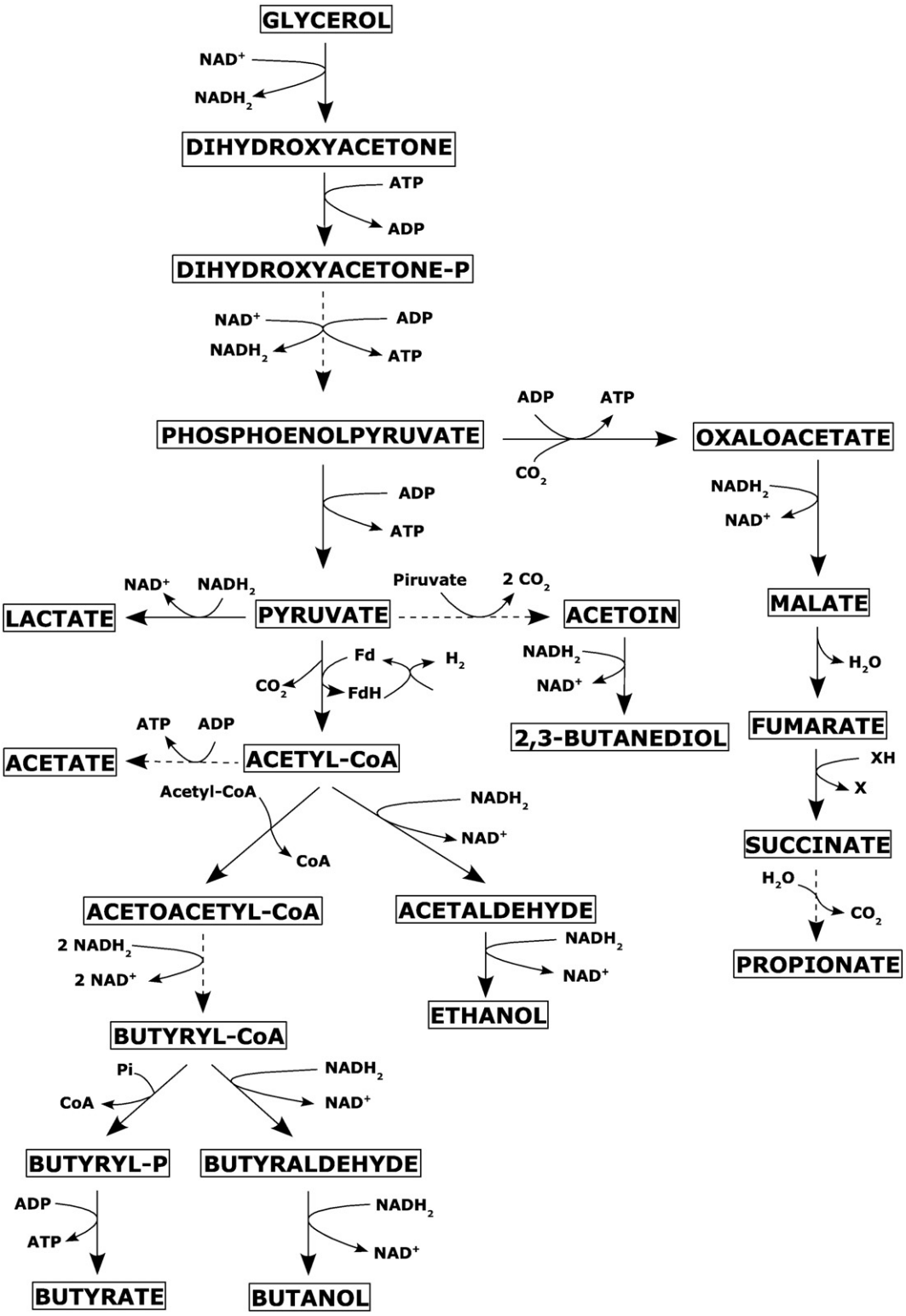

Fig. 4. Overview of some possible end products for different microorganisms during glycerol degradation.

temperature, high pressure and expensive catalysts (Lin et al., 2005) and produces toxic intermediates (Raynaud et al., 2003; González-Pajuelo et al., 2004, 2005a). Consequently, chemical synthesis is expensive and, thus, 1,3-PDO still has a low market volume (Deckwer, 1995). Because of the environmental benefits and use of a renewable feedstock, the biotechnological synthesis of 1,3-PDO appears to be an attractive alternative to chemical synthesis (Hao et al., 2006).

4.1.1. Bacterial production of 1,3-PDO

The biotechnological production of 1,3-PDO from glycerol has been demonstrated for several bacteria, such as *Lactobacillus brevis*, *L. buchnerii*, *Bacillus welchii*, *C. freundii*, *K. pneumoniae*, *C. pasteurianum*, *C. butyricum* and *E. agglomerans* (Nakas et al., 1983; Forsberg, 1987; Homann et al., 1990; Veiga da Cunha and Foster, 1992; Biebl, 1991; Biebl et al., 1992; Biebl and Marten, 1995; Daniel et al., 1995; Macis et al.,

1998; Deckwer, 1995; González-Pajuelo et al., 2006; Cheng et al., 2004; Yang et al., 2007). Complete conversion of glycerol to 1,3-PDO is not possible due to the requirement of an additional reducing equivalent (Chotani et al., 2000) (Fig. 2).

Glycerol fermentation by enterobacteriaceae results in the accumulation of two main products, 1,3-PDO and acetate, whereas the secondary products, lactate, formate, succinate and ethanol, are produced in variable amounts depending on the culture conditions (Homann et al., 1990; Barbirato et al., 1998). With *C. butyricum*, 1,3-PDO is the main product, with butyric acid and acetic acid as by-products in addition to CO_2 and H_2 (Barbirato et al., 1998). The bacterium *C. pasteurianum* growing on glycerol produces a variety of metabolic end products, such as *n*-butanol, 1,3-propanediol, ethanol, acetic acid, butyric acid and lactic acid (Biebl, 2001) (Fig. 4).

According to Biebl (1991), the concentrations that inhibit glycerol fermentation by *C. butyricum* are 60 g/L for 1,3-PDO, 27 g/L for acetate and 19 g/L for butyrate, and glycerol was supported until 80 g/L. Barbirato et al. (1998) report that, in batch fermentations using *C. butyricum* containing 112 g/L industrial glycerin, the final 1,3-PDO concentration was 63.4 g/L, which is close to the maximal concentration tolerated by this microorganism. The conversion yield was 0.69 mol/mol and maximal 1,3-PDO productivity was 1.85 g/L/h, with butyrate (7.5 g/L) and acetate (8.1 g/L) as the only by-products obtained.

Papanikolaou et al. (2000) investigated the production of 1,3-PDO by a newly isolated *C. butyricum* strain. The highest 1,3-PDO concentration obtained in continuous culture was 31–48 g/L, with a conversion yield of 0.55 g 1,3-PDO/g glycerol. Using a two-stage strategy, the maximum 1,3-PDO obtained was 41–46 g/L, with a maximum volumetric productivity of 3.4 g/L/h. Mu et al. (2006) used *K. pneumoniae* to ferment crude glycerol, obtaining a concentration of 51.3–53 g/L of 1,3-PDO with productivity of 1.7 g/L/h. The use of crude glycerol should simplify the process and reduce operational costs. In fed-batch fermentation with sucrose as a co-substrate, Yang et al. (2007) obtained up to 83.56 g 1,3-PDO/L with a yield of 0.62 mol/mol glycerol and productivity of 1.61 g/L/h with *K. oxytoca* M5a1, a mutant strain deficient in lactic acid biosynthesis. In the first report on pilot-scale production of 1,3-PDO using *K. pneumoniae* M5a1, Cheng et al. (2007) obtained 58.8 g 1.3-PDO/L, a yield of 0.53 mol/mol glycerol and productivity of 0.92 g/L/h.

Metabolic engineering can be used to manipulate the pathways so that the formation of by-products is reduced or even eliminated (Cheng et al., 2005, 2006). The genetic manipulation of the microorganisms can also eliminate pathways that compete with the desired product or enhance the production of the desired metabolites. The biological production of both glycerol and 1,3-propanediol is known, but it has never been demonstrated that a single organism can accomplish the entire process (Chotani et al., 2000). Thus, no natural microorganisms have yet been found that can directly convert glucose to 1,3-PDO (Hartlep et al., 2002). Moreover, wild-type *E. coli* strains do not ferment glycerol to 1,3-PDO (Cameron et al., 1998). Some studies have developed recombinant microorganisms for 1,3-PDO production from glucose. Hartlep et al. (2002) present a two-stage strategy for the conversion of glucose to 1,3-PDO. First, a recombinant *E. coli* strain produces glycerol from D-glucose. Then glycerol is converted to 1,3-PDO by *K. pneumoniae*. This two-stage process renders up to 60–70 g/L 1,3-PDO/L. In the DuPont and Genencor patent (Chotani et al., 2000), a process using a recombinant *E. coli* strain containing the genes from *S. cerevisiae* for glycerol production and the genes from *K. pneumoniae* for 1,3-PDO production is described (U.S. Patent 5,599,689, 1997). This recombinant microorganism reached a final 1,3-PDO concentration of 135 g/L using glucose as substrate; the productivity was 3.5 g/L/h and the efficiency of substrate conversion was 51% (Sanford et al., 2004). The need to add large amounts of expensive vitamin B_{12} (Raynaud et al., 2003) is a major limitation and has prevented the large-scale production of 1,3-PDO using this fermentation process (Yang et al., 2007).

Strains of *E. coli* that are able to convert glycerol to 1,3-PDO have been constructed by overexpressing genes of the *dha* regulon from *K. pneumoniae* or *C. freundii*. However, the glycerol conversion is low. The main reason for this low yield is a number of toxic by-products, such as glycerol-3-phosphate (Zhu et al., 2002). González-Pajuelo et al. (2005b) developed a recombinant *C. acetobutylicum* DG1 strain by introducing a plasmid (pSPD5) that contained the 1,3-propanediol pathway from *C. butyricum*. This bacterium is considered the best natural producer in terms of both yield and titer of 1,3-PDO produced. Recombinant *C. acetobutylicum* DG1 (pSPD5) produced 1104 mM 1,3-PDO in a fed-batch culture, with a yield of 0.65 mol/mol glycerol consumed and productivity of 1.70 g/L/h. The authors conclude that *C. acetobutylicum* DG1 (pSPD5) can be used for the continuous industrial production of 1,3-PDO with high yields, titers and productivity from raw glycerol from biodiesel (González-Pajuelo et al., 2005b, 2006). Zhang et al. (2006a) constructed a recombinant *E. coli* JM109 strain (pHsh-*dhaB-yqhD*) containing the genes *yqhD* (coding for 1,3-propanediol oxidoreductase from wild-type *E. coli*) and *dhaB* from *C. freundii* in the temperature control expression plasmid vector pHsh. This recombinant strain produced up to 41.1 g/L of 1,3-PDO in an optimized culture medium containing 61.8 g glycerol/L. Cameron et al. (1998) describe several metabolic engineering approaches for 1,2 and 1,3-propanediol production.

2,3-Butanediol is another glycol that can be produced from glycerol. According to Syu (2001), 2,3-butanediol is a compound that can be added as a flavoring agent in food products when converted to diacetyl through oxidation. 2,3-Butanediol can also be converted to 1,3-butadiene, which is used in the production of synthetic rubber. Many other derivatives of 1,3-butadiene are of commercial value, such as antifreeze agents, solvents and plastics, liquid fuel additives and polyurethanes for drugs, cosmetic products and lotions. The mechanisms that lead to the production of 1,3-PDO and 2,3-butanediol from glycerol by *K. pneumoniae* were discussed by Biebl et al. (1998) and the highest 2,3-butanediol production occurs at low pH and an excess of glycerol.

4.2. Dihydroxyacetone

Dihydroxyacetone is frequently used in the cosmetic industry and serves as a versatile building block for the organic synthesis of a variety of fine chemicals (Hekmat et al., 2003; Bauer et al., 2005). Due to rigorous safety requirements, the chemical production of DHA is rather expensive. Thus, DHA synthesis is performed more economically using a microbial process (Hekmat et al., 2003). It is produced via the oxidation of glycerol using the acetic acid bacterium *G. oxydans* (Flickinger and Perlman, 1977; Nabe et al., 1979; Wethmar and Deckwer, 1999; Bauer et al., 2005) in a process that requires good oxygenation and a medium containing yeast extract (Flickinger and Perlman, 1977). Wethmar and Deckwer (1999) developed a semi-synthetic medium that allows increased specific DHA production, significantly reducing the yeast extract in the medium.

There are only two pathways for glycerol catabolism in *G. oxydans* (Bories et al., 1991; Claret et al., 1994). Microbial growth is ensured by a cytoplasmic pathway, in which glycerol is phosphorylated to glycerol-3-phosphate and then dehydrogenated to DHA-phosphate (DHA-P), which is ATP- and NAD-dependent. DHA-P is catabolized in the pentose-phosphate pathway. DHA production occurs via a membrane-bound glycerol dehydrogenase, which appears to be the only process responsible for DHA synthesis and employs oxygen as the final acceptor of reduced equivalents, without NADH involvement (Fig. 3).

One of the main problems of microbial DHA synthesis is the fact that both the substrate and product have an inhibitory effect on bacterial growth (Bories et al., 1991; Claret et al., 1994; Hekmat et al., 2003; Bauer et al., 2005). Hekmat et al. (2003) propose a repeated-fed-batch mode, allowing a fully automated quasi-continuous operation that can be maintained over a period of several months with no decrease in productivity, thereby reducing processing costs. The productivity of the

process was increased by 75%, from 1.6 kg/m^3/h to about 2.8 kg/m^3/h, but the authors observed that maximum productivity had not yet been achieved. Using this new process, Bauer et al. (2005) demonstrated that the culture was able to grow in up to a DHA concentration of 80 kg/m^3 without any influence of product inhibition. *G. oxydans* lost its regeneration capability at DHA concentrations above 160 kg/m^3, but product formation was observed up to a maximum DHA concentration of 220 kg/m^3 because intact membrane-bound glycerol dehydrogenase was still active both in the irreversibly growth-inhibited cells as well as in the cell debris.

Gätgens et al. (2007) assessed the effect of the overexpression of glycerol dehydrogenase (ORFs *sldAB*) on glycerol oxidation, demonstrating that growth on glycerol was significantly improved in the overexpression strains (OD 2.8–2.9) compared to the control strains (OD 1.8–2.0). Both the DHA formation rate and the final DHA concentration were affected such that up to about 30 g/L of DHA was accumulated by the overexpression strains, compared to 18–25 g/L in control strains when 50 g/L glycerol was supplied. The higher concentration of enzyme possibly reduced the velocity of total glycerol dehydrogenase inactivation and slowed down the inactivation of glycerol oxidation and cell viability. This also explains the higher DHA production rate in the strains overexpressing the *sldAB* gene.

4.3. Succinic acid

Succinate has a specialty chemical market in industries producing food and pharmaceutical products, surfactants and detergents, green solvents, biodegradable plastics and ingredients to stimulate animal and plant growth. Due to its structure as a linear saturated dicarboxylic acid, succinate can be used as an intermediate chemical and be converted to 1,4-butanediol, tetrahydrofuran, γ-butyrolactone, adipic acid, *n*-methylpyrrolidone and linear aliphatic esters (Zeikus et al., 1999). An increasing demand for succinic acid is expected as its use is extended to the synthesis of biodegradable polymers such as polybutyrate succinate (PBS) and polyamides (Song and Lee, 2006). Ranucci et al. (2000) describe the synthesis of a new biodegradable polymer, poly(1,3-propylene succinate), obtained through the thermal polycondensation of succinic acid with 1,3-PDO.

Succinate is currently produced petrochemically from butane through maleic anhydride; only natural succinic acid sold in the food market is produced by fermentation (Zeikus et al., 1999). Succinate is normally produced under anaerobic conditions through several different metabolic pathways, such as PEP and pyruvate carboxylation. *Anaerobiospirillum succiniciproducens* is one of the most efficient succinate producers and uses the PEP carboxylation pathway (Fig. 4), catalyzed by PEP carboxykinase (or PEP carboxylase), malate dehydrogenase, fumarase and fumarate dehydrogenase (Lee et al., 2004).

Lee et al. (2001) showed that *A. succiniciproducens* can efficiently convert glycerol to succinate. A maximum of 19 g/L of succinic acid was obtained by fermentation of *A. succiniciproducens* when glycerol was used as the sole carbon source in a medium supplemented with yeast extract and 29.6 g/L when glycerol was fed with glucose. According with the authors, succinic acid production from glycerol presents several advantages over glucose, such as high succinic acid yield with reduced acetic acid formation. This is advantageous because acetic acid imposes difficulties with respect to downstream processes for the recovery of succinic acid. Considering the costs of separation and purification of succinate from fermentation broth containing mixed acids, the formation of by-products is a problem to be solved through metabolic engineering and fermentation process optimization (Song and Lee, 2006).

Fumaric acid is a chemical product that has several industrial applications, such as an acidulant in the food industry, the manufacturing of sizing resins for the paper industry and is a promising candidate in the obtainment of polymers (Zhou et al., 2002). While no work has been performed to evaluate the production of fumaric acid from glycerol, there is a possibility of it also being obtained from glycerol fermentation, as it is a direct precursor of succinic acid (Fig. 4).

4.4. Propionic acid

Propionic acid is another substance synthesized in a similar pathway to that of succinic acid and is derived directly from a metabolic pathway balanced with regard to redox-equivalents (Barbirato et al., 1997a) (Fig. 4). Propionate is used as an antifungal agent in food and feed and as a basic chemical to produce cellulose-based plastics, herbicides, solvents and perfumes (Barbirato et al., 1997a), arthritis drugs, flavors and thermoplastics (Himmi et al., 2000). The numerous industrial applications of propionic acid account for an increasing interest in the development of a biotechnological production process based on the renewable resource glycerol (Barbirato et al., 1997a, Himmi et al., 2000).

Barbirato et al. (1997a) assessed the production of propionate from glycerol by three bacterial strains: *Propionibacterium acidipropionici*, *Propionibacterium acnes* and *Clostridium propionicum*. Considering fermentation time and conversion yield, the best strain for glycerol conversion to propionate was *P. acidipropionici*. The fermentation profile of this bacterium revealed five end products, consisting of propionic acid as the major product (0.84 mol/mol and productivity of 0.18 g/L/h), with the following minimal by-products: succinate, acetate, formate and *n*-propanol. Productivity of up to 0.36 g/L/h was obtained and the maximal propionic acid concentration was 42 g/L using 80 g/L glycerol in the medium. Acetic acid formation was low when compared to the amount observed during glucose fermentation by *P. acidipropionici*. As the efficiency of propionic acid extraction through distillation is strongly limited by acetic acid (as can be observed for succinic acid), the extremely low acetic acid concentration obtained by using glycerol as substrate should greatly increase the yield of propionic acid recovered through distillation and simplify the distillation procedure (Barbirato et al., 1997a). The authors conclude that glycerol is a promising substrate for propionic acid production both in terms of conversion yield and productivity, which is similar or superior to that of lactic acid (0.35 g/L/h) or glucose (0.28 g/L/h). Better efficiency for propionic acid production from glycerol could be expected because of its higher reduction level over conventional substrates.

4.5. Ethanol

Ethanol is primarily produced from sugarcane in Brazil, from corn starch in the USA and from sugar beets in the EU. In a study to obtain solvents from algal biomass, Nakas et al. (1983) describe a soil bacterium tentatively classified as a member of the genus *Bacillus*, which produces ethanol (final concentration 7.0–9.6 g/L) from a glycerol-enriched algal mixture. Jarvis et al. (1997) demonstrated that formate and ethanol are the major products of glycerol fermentation by *Klebsiella planticola* isolated from the rumen. These authors state "the observation that formate and ethanol are the major glycerol fermentation products is an interesting one with respect to microbiological ecology of the red deer rumen". With the expected surplus of glycerol from biodiesel, this observation will become interesting also to industrial microbiology, demonstrating the importance of basic studies in microbial ecology and diversity to the development of applied microbiology.

Dharmadi et al. (2006) report that *E. coli* can ferment glycerol in a pH-dependent manner, being linked to the availability of CO_2, which is produced under acidic conditions by the oxidation of formate by the enzyme formate hydrogen lyase. Glycerol (10 g/L) was almost completely consumed within 84 h, with ethanol (86%) and succinic acid (7%) accounting for 93% (molar basis) of the products. Only minor amounts of acetate were produced. According the authors, *E. coli* is

already a good biocatalyst for the conversion of glycerol into ethanol and hydrogen. Ito et al. (2005) state that *E. aerogenes* can be used for the high-yield production of ethanol from biodiesel wastes containing glycerol. In a synthetic medium containing biodiesel wastes with glycerol at 80 mM, glycerol was consumed in 24 h, yielding H_2 at 0.89 mol/mol glycerol and ethanol at 1.0 mol/mol glycerol. The ethanol yield from glycerol fermentation described is low and future development is needed in order for this fermentation to be applicable.

4.6. Citric acid

Citric acid is produced in large quantities by fermentation, with a global production rate estimated at about 1.4 million tons in 2004. Citric acid is widely used to impart a pleasant, tart/fruity flavor to foods and beverages and is also used as an additive in detergents, pharmaceuticals, cosmetics and toiletries (Soccol et al., 2006). In general, citric acid is produced by submerged microbial fermentation on molasses using *Aspergillus niger*. In recent years, considerable interest has arisen in finding less expensive carbon sources for citric acid production (Soccol et al., 2006). Papanikolaou et al. (2002) found that the citric acid producer *Yarrowia lipolytica* grows on glycerol, concluding that raw glycerol may be a suitable substrate for citric acid production. This yeast produced up to 35 g/L of citric acid when a high initial concentration of glycerol was used in the culture medium. Growth and citric acid production parameters on glycerol were similar to those obtained using glucose. Imandi et al. (2007) describe a statistically optimized medium for *Y. lipolytica* NCIM 3589. The authors predict a final citric acid concentration of 77.4 g/L in the fermentation broth when using raw glycerol as a substrate.

4.7. Pigments

Some studies have been made using glycerol as carbon source for pigment production, such prodigiosin and astaxantin. Prodigiosin is a deep red pigment formed by the Gram-negative bacterium *Serratia marcescens* when cultivated on rich solid media. This pigment is an immunosupressor and has been reported to induce apoptosis of several cancer cell lines, such as hematopoietic cancer cells, colon cancer cells, B-lymphatic cancer cells and chronic lymphocytic leukemia cells, but not in nonmalignant cells, which indicates that prodigiosin may have potential as new antineoplastic candidate (Montaner et al., 2000; Campàs et al., 2003). Tao et al. (2005) demonstrated that glycerol allowed the highest prodigiosin production rate when used as main carbon source in the medium among several others carbon sources. In a two-step feeding strategy using glucose for growth and then glycerol for prodigiosin synthesis, a *S. marcescens* mutant produced about 583 mg prodigiosin/L in 30 h, with glycerol as the sole carbon source in a 5-l bioreactor.

Astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) is a red or orange pigment found in marine environments. It is used to feed animals such as salmon, trout (freshwater) and crustaceans in order to give them a color that appeals to consumers. Synthetic astaxanthin is currently incorporated at a rate of approximately 40–150 mg/kg of feed. However, a natural source of astaxanthin is preferred for an apparent safe utilization as a food additive (Kusdiyantini et al., 1998). The yeast *Phaffia rhodozyma* produces astaxanthin and it is considered a potential source of this pigment. Kusdiyantini et al. (1998) demonstrated that glycerol is a potential substrate for astaxanthin production in *P. rhodozyma*, obtaining a maximum total volumetric astaxanthin concentration of 33.7 mg/L.

4.8. Polyhydroxyalcanoate

Polyhydroxyalcanoate (PHA) represents a complex class of naturally occurring bacterial polyesters that are synthesized intracellularly as carbon and energy reserve materials (Ashby et al., 2004) by microorganisms belonging to the Bacteria and Archaea domains of life (Solaiman et al., 2006). These reserve materials are synthesized when the microorganisms are grown under nutrient-limited conditions (Ashby et al., 2005). Since PHAs are biodegradable and biocompatible, they may replace petroleum-derived polymers, which are widely used in medicine, drug delivery, agriculture/horticulture, the fiber industry and the consumer care branch (Solaiman et al., 2006).

Due to the cost of the fermentative substrate, research efforts abound to exploit inexpensive fermentable raw material as fermentative substrates for PHA production (Solaiman et al., 2006). Bormann and Roth (1999) describe the production of polyhydroxybutyrate (PHB) by *Methylobacterium rhodesianum* and *Ralstonia eutropha* in a medium containing glycerol and casein peptone or casamino acids, but the conversion was low. Ashby et al. (2004) used *Pseudomonas* sp. for PHA production from a co-product stream of biodiesel containing glycerol, free fatty acids and fatty acid methyl ester and showed that glycerol was the preferred substrate by *P. oleovorans*. This is interesting in that glycerol is the most energetically favorable substrate for the formation of acetyl-CoA, the precursor for PHB synthesis. The authors conclude that co-product from biodiesel can be used for PHA production without the need for separating and recovering glycerol and other components. Ashby et al. (2005) showed that up to 5% glycerol (v/v) could be used for the production of both PHB by *P. oleovorans* and medium-chain-length PHA by *P. corrugata* under identical growth conditions, allowing mixed culture fermentations to be used in the formation of PHA polymer blends with varying blend ratios.

Koller et al. (2005) obtained 16.2 g/L of PHA in the glycerol liquid phase from the biodiesel industry, which contained about 70% glycerol (w/w). PHB from glycerol has low molecular weight, because the substrate causes termination of chain propagation through covalent esterification of glycerol to PHB in a chain terminating position, thus rendering PHAs of low molecular weight (Ashby et al., 2005; Koller et al., 2005). This finding is certain to have an important implication when using glycerol to produce PHA (Solaiman et al., 2006).

4.9. Biosurfactants

Research in the field of biosurfactants has expanded quite a lot in recent years, due to their potential use in areas such as the food industry, agriculture, pharmaceutical industry, crude oil recovery and bioremediation of contaminated sites (Bannat et al., 2000). The development of studies on biosurfactants has a considerable importance, mainly in view of the present concern for protecting the environment. Biosurfactants have many advantages over chemically manufactured surfactants. They are less toxic, biodegradable and have unique surface-active properties (Bognolo, 1999). Nitschke et al. (2005) found that *Pseudomonas aeruginosa* can synthesize rhamnolipids from glycerol as the sole carbon source, but the yield was low when compared to using hydrophobic substrates. Zhang et al. (2005) produced 15.4 g/L rhamnolipids using *P. aeruginosa* growing on a basal mineral medium containing glycerol as the sole carbon source. Rahman et al. (2002) observed that *P. aeruginosa* DS10-129 produced 1.77 g/L rhamnolipids on glycerol. These results show that glycerol can be used for the production of biosurfactants.

5. Conclusions

In general, the use of renewable waste substrates is an environmental-friendly choice that also contributes to the reduction of waste treatment costs and increases the economic value of by-products. Some chemical commodities currently produced from petroleum can, in principle, be produced biotechnologically from glycerol using microorganisms. This bioconversion would directly benefit the environment by obtaining biodegradable polymers, promoting the use of biodiesel and reducing petroleum dependency. The development of processes for converting inexpensive glycerol into higher value products is expected

to make biodiesel production more economical and will thus help establish more biorefineries (Dharmadi et al., 2006). This will also have an important social impact, as small farmers cultivate oleaginous plants which in turn are the basis for biodiesel production. Future research will focus on the isolation and characterization of microorganisms that can use glycerol as a carbon source and generate valuable molecules with unusual properties, thereby broadening the potential applications of this cheap by-product of transesterification.

Glycerol is a versatile carbon and energy source with many possible applications in industrial fermentation. Most research has focused on the employment of glycerol in solvent production such as dihydroxyacetone and 1,3-propanediol. However, glycerol can also be used as a carbon source to obtain other valuable microbial products, such as recombinant proteins and enzymes, medicinal drugs, antibiotics and fine chemicals.

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