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Glycerol production by microbial fermentation: A review

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Abstract

Microbial production of glycerol has been known for 150 years, and glycerol was produced commercially during World War I. Glycerol production by microbial synthesis subsequently declined since it was unable to compete with chemical synthesis from petrochemical feedstocks due to the low glycerol yields and the difficulty with extraction and purification of glycerol from broth. As the cost of propylene has increased and its availability has decreased especially in developing countries and as glycerol has become an attractive feedstock for production of various chemicals, glycerol production by fermentation has become more attractive as an alternative route. Substantial overproduction of glycerol by yeast from monosaccharides can be obtained by: (1) forming a complex between acetaldehyde and bisulfite ions thereby retarding ethanol production and restoring the redox balance through glycerol synthesis; (2) growing yeast cultures at pH values near 7 or above; or (3) using osmotolerant yeasts. In recent years, significant improvements have been made in the glycerol production using osmotolerant yeasts on a commercial scale in China. The most outstanding achievements include: (1) isolation of novel osmotolerant yeast strains producing up to 130 g/L glycerol with yields up to 63% and the productivities up to 32 g/(L day); (2) glycerol yields, productivities and concentrations in broth up to 58%, 30 g/(L day) and 110–120 g/L, respectively, in an optimized aerobic fermentation process have been attained on a commercial scale; and (3) a carrier distillation technique with a glycerol distillation efficiency greater than 90% has been developed. As glycerol metabolism has become better understood in yeasts, opportunities

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will arise to construct novel glycerol overproducing microorganisms by metabolic engineering.
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1. Introduction

Glycerol, a 1,2,3-propanetriol, is a simple alcohol with many uses in the cosmetic, paint, automotive, food, tobacco, pharmaceutical, pulp and paper, leather and textile industries (Table 1) or as a feedstock for the production of various chemicals. Glycerol is also known as glycerin or glycerine. Glycerol has also been considered as a feedstock for new industrial fermentations in the future. For example, glycerol can be fermented to 1,3 propanediol (Biebl et al., 1998, 1999), which is used for the chemical synthesis of poly(trimethylene terephthalate), a new polyester with novel fiber and textile applications that combines excellent properties (good resilience, inherent stain resistance, low static generation) with an environmentally benign manufacturing process (Biebl et al., 1998, 1999). The transformation of glycerol to dihydroxyacetone by the bacterium *Acetobacter suboxidans* is another example of a potential process (Charney, 1978). In a submerged fermentation, the bacteria produce dihydroxyacetone in yields of 75–90% from a 5–15% solution of glycerol. The dihydroxyacetone can be transformed further by a dihydroxyacetone kinase to dihydroxyacetone phosphate, which serves as an essential substrate for some aldolases to produce various optically active sugar derivatives (Itoh et al., 1999).

Glycerol can be produced either by microbial fermentation or by chemical synthesis from petrochemical feedstocks or can be recovered as a by-product of soap manufacture from fats. Traditionally, glycerol is produced as a by-product of the hydrolysis of fats in soap and other related materials and contributes significantly to the present glycerol production volume of about 600 000 tons annually. This process is now of lesser importance in industrial nations and many developing countries, because of the replacement of soap with detergents (Rehm, 1988; Agarwal, 1990). Currently, approximately 25% of world glycerol production occurs by the oxidation or chlorination of propylene to glycerol, but this route has declined in relative importance since the early 1970s (Hester, 2000) partially because of environmental concerns. Furthermore, as the cost of propylene has increased and its availability has decreased especially in developing countries, glycerol production by fermentation has become more attractive as an alternative route (Agarwal, 1990; Wilke, 1999). A significant amount of glycerol is also synthesized from allyl alcohol. Currently, the price of glycerol is between US\$1.10/kg and US\$1.25/kg and is expected to increase in line with inflation over the next 10 years (Hester, 2000). Glycerol production costs by microbial fermentation are difficult to estimate. Recently, High Plains Corporation (Wichita, KS) reported that glycerol production costs between US\$0.40/kg and US\$0.53/kg would result in a profitable operation.

Table 1
Current uses of glycerol^a

| Use field | Use (%) | | | |
|--------------------|----------------------------|-------------------------------|-----------------------------|-----------------------------|
| | USA (160 000 tons/year) | Europe (190 000 tons/year) | Japan (50 000 tons/year) | China (80 000 tons/year) |
| Drugs | 39.5 | 23.1 | 34.0 | 5.2 |
| Tobacco | 15.8 | 2.5 | 5.3 | 7.3 |
| Glycerintriacetate | ND ^b | 14.4 | ND | ND |
| Food | 14.5 | 5.6 | ND | ND |
| Polyether alcohol | 10.5 | 13.1 | 11.6 | 5.2 |
| Paints | 9.2 | 13.1 | 19.5 | 49.0 |
| Cellophane | 2.0 | 4.4 | 3.8 | 1.5 |
| Dynamite | 0.6 | 3.1 | 1.9 | 3.1 |
| Toothpaste | ND | ND | ND | 16.0 |
| Cosmetics | ND | ND | ND | 6.3 |
| Miscellaneous | 7.9 | 20.6 | 23.9 | 7.2 |

^a The data are mainly from the reports from Chinese Chemical Engineering Information Center (unpublished data).

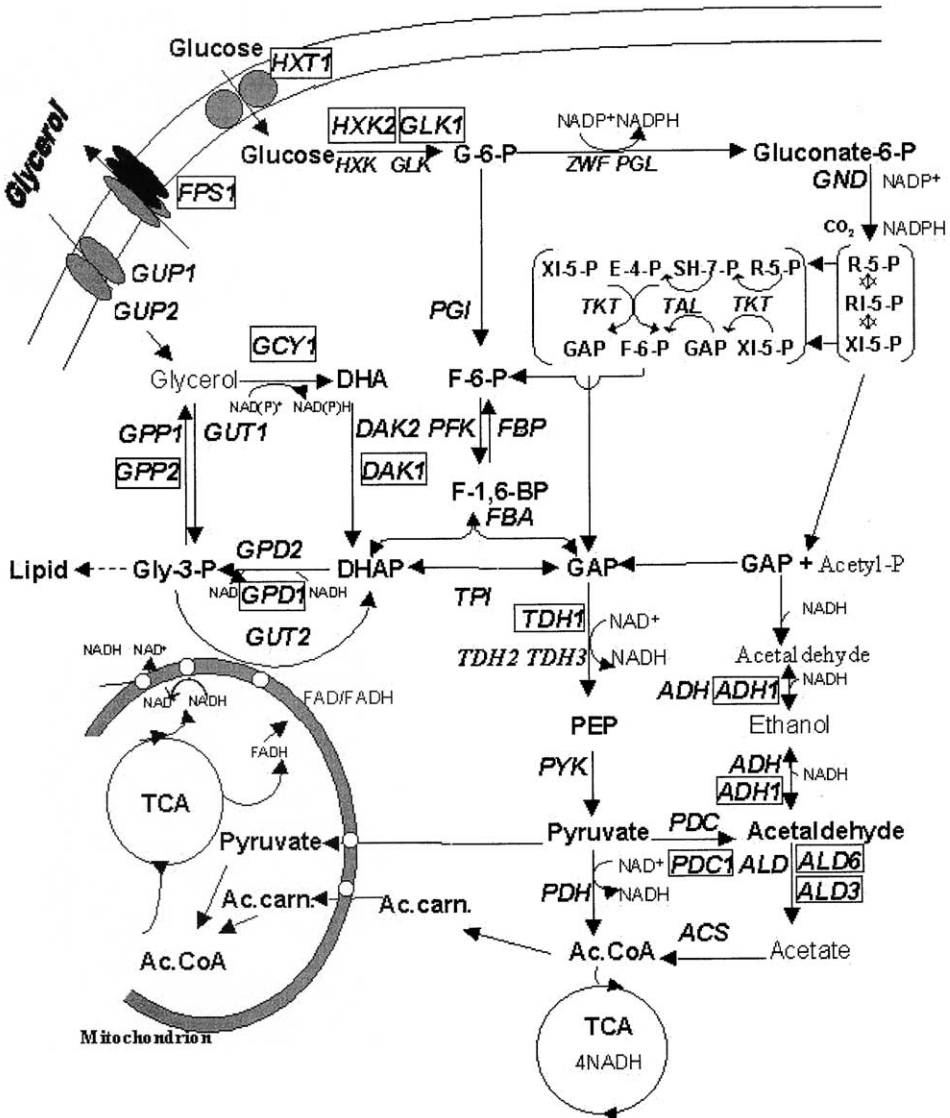
^b ND = no data.

Glycerol was produced via the fermentation route for the first time on a large scale using the sulfite-steered yeast process during World War I when demand for glycerol in explosive manufacture exceeded the supply from the soap industry (Prescott and Dunn, 1959). However, wartime process technology could never adapt to the peacetime competition from the chemically synthesized process developed after World War II as yields of glycerol from sugar by fermentation were low and recovery by distillation was inefficient.

Glycerol production by yeast fermentation has been known since the investigations of Pasteur (1858). In *Saccharomyces cerevisiae*, glycerol is a by-product of the fermentation of sugar to ethanol in a redox-neutral process. The role of NADH-consuming glycerol formation is to maintain the cytosolic redox balance especially under anaerobic conditions, compensating for cellular reactions that produce NADH (van Dijken and Scheffers, 1986). Substantial overproduction of glycerol from monosaccharides can be obtained by yeast: (1) forming a complex of acetaldehyde with the bisulfite ion that limits ethanol production and that promotes reoxidation of glycolytically formed NADH by glycerol synthesis; (2) growing at pH values around 7 or above; and (3) by using osmotolerant yeasts (Rehm, 1988; Agarwal, 1990).

The purpose of this review is to examine recent developments in the production of glycerol by microorganisms. In the review, we will examine the significant improvements in the understanding of glycerol synthesis and export by yeast. Other aspects that will be covered are the novel microorganisms used to produce glycerol, the fermentation and glycerol recovery processes currently available and the manipulation of glycerol producing pathways by environmental factors and strain engineering. Prior to 1990, this topic of glycerol production was reviewed by Underkofler (1954), Bernhauer (1957), Weixl-

Hofmann and von Lacroix (1962), Onishi (1963), Spencer and Spencer (1978), Rehm (1967, 1980, 1988), Vijaikishore and Karanth (1984, 1986b) and Agarwal (1990). The reader is also referred to recent reviews on glycerol metabolism in yeasts that do not cover the biotechnological aspects (Prior and Hohmann, 1997; Nevoigt and Stahl, 1997; Wang and Zhuge, 1999b; Zhuge and Wang, 1999). Glycerol production by bacteria and algae will only be mentioned briefly.



2. Glycerol metabolism in yeast

2.1. Glycerol synthesis

2.1.1. Via glycerol-3-phosphate

Glycerol is synthesized in the cytosol of the yeast *S. cerevisiae* from the glycolytic intermediate dihydroxyacetone phosphate in two steps that are catalyzed by glycerol-3-phosphate dehydrogenase (Gpd) and glycerol-3-phosphatase (Gpp), respectively (Fig. 1). Each enzyme has two isoenzymes, the osmotically induced Gpd1p, the constitutive Gpd2p (Albertyn et al., 1994; Ansell et al., 1997; Bjorkqvist et al., 1997; Ohmiya et al., 1995, 1997), the osmotic-induced Gpp2p and the constitutive Gpp1p (Norbeck et al., 1996). The *GPD1* and/or *GPD2* genes encoding glycerol-3-phosphate dehydrogenase have been cloned and sequenced from *S. cerevisiae* (Larsson et al., 1993; Albertyn et al., 1994; Eriksson et al., 1995), *Saccharomyces diastaticus* (Wang et al., 1994), *Schizosaccharomyces pombe* (Ohmiya et al., 1995), *Candida glycerinogenes* (Wang and Zhuge, 1999a) and *Zygosaccharomyces rouxii* (Genbank Accession Numbers AJ251481, AB047394, AB047395). This, together with the report of enzyme glycerol-3-phosphate dehydrogenase activity in *Debaryomyces hansenii* (Nilsson and Adler, 1990), suggests that synthesis of glycerol via glycerol-3-phosphate is fairly common in yeasts. Of the two enzymes, Gpd1p is the key enzyme in glycerol formation in *S. cerevisiae*. Glycerol-3-phosphate and dihydroxyacetone phosphate are also important metabolic intermediates for synthesis of other substances besides glycerol. For example, glycerol-3-phosphate and dihydroxyacetone phosphate can serve as precursors to synthesize phospholipids and glycerolipids (Racenis et al., 1992; Daum et al., 1998; Athenstaedt et al., 1999) and dihydroxyacetone phosphate is also a precursor for amino acid synthesis.

2.1.2. Other routes of synthesis

Whether an alternative route for the synthesis of glycerol exists in yeasts is unclear at present as experimental data supporting such a possibility is lacking. An alternative

Fig. 1. Biochemical pathways of glycerol metabolism in yeast. *ACS*: acetyl CoA synthetase; *ADH*: alcohol dehydrogenase; *ALD*: aldehyde dehydrogenase; *DAK*: dihydroxyacetone kinase; *FBA*: fructose biphosphate aldolase; *FBP*: fructose biphosphatase; *FPS*: glycerol facilitator; *GCY*: glycerol dehydrogenase; *GLK*: glucokinase; *GND*: 6-phosphogluconate dehydrogenase; *GPD*: cytoplasmic glycerol-3-phosphate dehydrogenase; *GPP*: glycerol-3-phosphatase; *GUP*: glycerol uptake protein; *GUT1*: glycerol kinase; *GUT2*: mitochondrial glycerol-3-phosphate dehydrogenase; *HXX*: hexokinase; *HXT*: hexose transferase; *PDC*: pyruvate decarboxylase; *PDH*: pyruvate dehydrogenase; *PFK*: fructose-6-phosphate kinase; *PGI*: glucose-6-phosphate isomerase; *PGL*: 6-phosphogluconolactonase; *PYK*: pyruvate kinase; *TAL*: transketolase; *TKT*: transaldolase; *TDH*: glyceraldehyde-3-phosphate dehydrogenase; *TPI*: triose phosphate isomerase; *ZWF*: glucose-6-phosphate dehydrogenase. Ac.carn. = Acetylcarnitine; Ac.CoA = Acetyl-coenzymeA; DHA = Dihydroxyacetone; DHAP = Dihydroxyacetone phosphate; E-4-P = Erythrose-4-phosphate; F-6-P = Fructose-6-phosphate; F-1,6-BP = Fructose-1,6-biphosphate; GAP = Glyceraldehyde-3-phosphate; Gly-3-P = Glycerol-3-phosphate; G-6-P = Glucose-6-phosphate; PEP = Phosphoenolpyruvate; R-5-P = Ribulose-5-phosphate; RI-5-P = Ribose-5-phosphate; SH-7-P = Sedoheptulose-7-phosphate; TCA = Tricarboxylic acid cycle; XI-5-P = Xylulose-5-phosphate. Blocks indicate the proteins encoded by the genes regulated by osmotic stress.

route apparently does not exist in *S. cerevisiae* as the *gpd1Δ gpd2Δ* double mutant strain fails to accumulate intracellular glycerol (Ansell et al., 1997). This strain is able to grow on glucose (Bjorkqvist et al., 1997; Nissen et al., 2000), but, evidently, the failure to produce glycerol-3-phosphate as a lipid precursor does not prevent cell growth as dihydroxyacetone phosphate can replace glycerol-3-phosphate (Athenstaedt et al., 1999).

2.2. Glycerol dissimilation

Glycerol may be dissimilated via glycerol-3-phosphate or dihydroxyacetone by yeast.

2.2.1. Via glycerol-3-phosphate

In *S. cerevisiae*, glycerol is dissimilated by glycerol kinase encoded by *GUT1* (Sprague and Cronan, 1977; Pavlik et al., 1993) and a specific flavin adenine dinucleotide (FAD)-dependent and mitochondrion-located glycerol-3-phosphate dehydrogenase (Gut2p) encoded by *GUT2* (Fig. 1; Ronnow and Kielland-Brandt, 1993). Mutants defective in *GUT1* or *GUT2* are unable to utilize glycerol as carbon source (Sprague and Cronan, 1977; Pavlik et al., 1993; Ronnow and Kielland-Brandt, 1993). The product formed by glycerol kinase, glycerol-3-phosphate, can be used either as a precursor for lipid biosynthesis or for conversion to dihydroxyacetone phosphate. The latter intermediate can either be transformed to glyceraldehyde-3-phosphate by a triose phosphate isomerase into the center metabolic pathway or can serve as a substrate for the synthesis of other metabolites (Grauslund et al., 1999). Gpd1p, Gpd2p and Gut2p also contribute to the 'glycerol-3-phosphate shuttle' of yeast, which may play an important role during aerobic growth of *S. cerevisiae* (Larsson et al., 1998). This route of glycerol dissimilation has also been observed in a number of other yeasts such as *D. hansenii* (Adler et al., 1985), *Z. rouxii* (van Zyl et al., 1991), *C. glycerinogenes* (Wang et al., 2000) and *S. pombe* (May et al., 1982; Gancedo et al., 1986).

2.2.2. Via dihydroxyacetone

A number of yeasts are thought to dissimilate glycerol via dihydroxyacetone (Fig. 1). Initially, glycerol is oxidized to dihydroxyacetone by glycerol dehydrogenase and then phosphorylated to dihydroxyacetone phosphate by dihydroxyacetone kinase. In *S. cerevisiae*, the genes *GCY1* and *DAK1* or *DAK2* encoding the two steps of the pathway, respectively, are present (Norbeck and Blomberg, 1997), although the significance of the pathway is uncertain. It might play a role in the regulation of the glycerol concentration during hyperosmotic stress (Blomberg, 2000). In other yeasts, this pathway may be more significant. *DAK* genes from *S. pombe* (Kimura et al., 1998; Itoh et al., 1999) and *Z. rouxii* (Wang and Prior; GenBank accession number AJ2,94719) have been cloned and sequenced. Recently, the sequences of *GCY1* and *GCY2* from *Z. rouxii* have been deposited in GenBank (Accession Numbers AB047396 and AB047397). This evidence, together with reports on enzyme activities of glycerol dehydrogenase and dihydroxyacetone kinase in various yeasts (Babel and Hofmann,

1982; Adler et al., 1985; Kong et al., 1985; van Zyl et al., 1991), suggests that this pathway is found in many yeasts.

2.3. Glycerol flux across the membrane

The flux of glycerol across the plasma membrane of *S. cerevisiae* is controlled either by passive diffusion or by a channel protein (Fps1p; van Aelst et al., 1991; Luyten et al., 1995; Sutherland et al., 1997) or by an active uptake mechanism (Gup1p, Gup2p; Holst et al., 2000) (Fig. 1). Whereas glycerol permeation by passive diffusion occurs in both directions, it is thought that the Fps1p-channel protein is primarily important for the export of glycerol from the cell especially under fermentative conditions (Tamas et al., 1999). The presence of a channel in other yeasts to export glycerol is at present unknown although active uptake of glycerol has been described (Adler et al., 1985; van Zyl et al., 1990; Lages et al., 1999).

2.4. The function of glycerol in cell metabolism

2.4.1. Osmoregulation

Glycerol plays an essential role as a compatible solute during osmoregulation in yeasts (Blomberg and Adler, 1992). In response to decreased extracellular water activity, *S. cerevisiae* greatly increases its rate of glycerol formation (Blomberg and Adler, 1989). A marked rise in the level of cytoplasmic NAD⁺-dependent glycerol-3-phosphate dehydrogenase (Gpd1p) has been observed following osmotic shock (Edgley and Brown, 1983; Blomberg and Adler, 1989; Andre et al., 1991). The osmotic induction of Gpd1p occurs on a transcriptional level that is controlled by a specific signal transduction pathway (high osmolarity glycerol response pathway, HOG pathway) regulating *GPD1* and results in elevated formation of glycerol (Varela et al., 1992; Brewster et al., 1993; Albertyn et al., 1994).

The intracellular accumulation of glycerol in *S. cerevisiae* is controlled by the Fps1p-channel (Luyten et al., 1995). Under conditions of hyperosmotic stress, the channel is closed thereby conserving the glycerol within the cell in order to maintain an osmotic equilibrium with the external environment. In the absence of hyperosmotic stress, the channel remains open and glycerol freely permeates from the cell (Tamas et al., 1999; Hohmann et al., 2000).

2.4.2. Redox balancing

Glycerol is produced by *S. cerevisiae* during fermentation of glucose to ethanol in order to maintain the redox balance. The importance of genes *GPD2* and *GPPI* in cellular redox balancing was confirmed by the deletion of the genes. This results in a strain unable to grow in the absence of oxygen (Ansell et al., 1997; Bjorkqvist et al., 1997, Norbeck and Blomberg, 1997; Nissen et al., 2000). Attempts to increase glycerol production by *S. cerevisiae* by overexpressing the gene *GPD1* disturb the redox balance in the cell. This results in a marked increase in by-product synthesis such as pyruvate, acetate, acetoin, 2,3-

Table 2
Comparison of different processes used in glycerol production

| Item | Represented processes | | | Algal process |
|--|--|---|--|------------------------------|
| | Yeast processes | Alkali process | Osmotolerant yeast process | |
| Organisms | <i>S. cerevisiae</i> | <i>S. cerevisiae</i> | Osmotolerant yeasts | Halotolerant alga |
| Substrate | glucose | glucose | glucose/sucrose | CO ₂ , light |
| Steering agent needed such as sulfite | Yes | No | No | No |
| Oxygen relationship | Anaerobic | Anaerobic | Aerobic | Aerobic |
| Fermentation time | 4–5 days or longer | 5–7 days or longer | Usually 3–5 days | 10–14 days |
| Fermentation control | Difficult | Difficult | Easy | Difficult |
| Infection problems | Sometimes | Sometimes | Seldom | Seldom |
| By-products | Ethanol, acetaldehyde | Ethanol | None or other polyols | None |
| Average glycerol productivity | 15 g/(L day) | 9 g/(L day) | 28–32 g/(L day) | 66 mg/(L day) |
| Conversion efficiency (glycerol/ amount of sugar consumed) | ~25% | ~25% | ~60% | ND ^a |
| Concentration of glycerol in broth in batch fermentation | 30–40 g/L | 30–40 g/L | 110–130 g/L | 0.18 g/L |
| Environmental pollution | Heavy | Heavy | Light | None |
| Commercialization | Yes, but currently uneconomical | No and currently uneconomical | Yes and economical | No |
| Reference | Cocking and Lilly, 1919; Kalle et al., 1985; Harris and Hajny, 1960 | Neuberg and Hirsch, 1919a; Underkofler et al., 1951 | Onishi 1957; Zhuge, 1973; Zhuge and Fang, 1994 | Ben-Amotz and Avron, 1981 |

^a ND = no data.

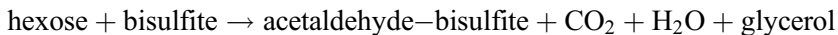
butanediol and succinate in order to maintain the redox balance (Michnick et al., 1997; Roustan et al., 1999).

3. Processes for glycerol production

The processes for glycerol production can be classified into anaerobic, aerobic and autotrophic processes and are defined by the substrate, oxygen relationships and whether steering agents are used or not (Table 2).

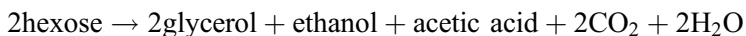
3.1. Production by *S. cerevisiae*

Substantial overproduction of glycerol by *S. cerevisiae* from monosaccharides can be achieved by combining acetaldehyde with the bisulfite ion (known as the steering agent) or by growing the cell at pH values around 7 or above. The first method, based on the trapping of acetaldehyde by bisulfite ions, yields the following reaction:



Acetaldehyde is unable to serve as an electron acceptor for cytosolic NADH and the accumulated NADH is instead oxidized by the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate (Neuberg and Farber, 1919a). Various salts have been used as steering agents such as sodium sulfate (Cocking and Lilly, 1919; Connstein and Ludecke, 1924; Harris and Hajny, 1960; Rapin et al., 1994), sodium sulfite and bisulfite (Freeman and Donald, 1957a), ammonium sulfite (Fulmer et al., 1945) and magnesium/calcium sulfite (Fulmer et al., 1947). In some instances, the fermentation process using the steering agent has been adapted to improve the efficiency of glycerol production (Table 3). Examples are fermentation under constant vacuum/CO₂ sparging (Kalle and Naik, 1985; Kalle et al., 1985), using immobilized cells in a semicontinuous or continuous processes (Bisping and Rehm, 1986; Vijaikishore and Karanth, 1986a; Bisping et al., 1989; Hecker et al., 1990) and a combined osmotic and sulfite stress process (Petrovska et al., 1999).

The second method, operated at pH values of 7 or above, is based on the reaction with the stoichiometry:



This reaction occurs in parallel with the normal alcoholic fermentation (Neuberg and Hirsch, 1919a,b). Acetic acid is produced from acetaldehyde due to the increased activity of aldehyde dehydrogenase, whose optimal pH is 8.75 (Black, 1951). This oxidation generates a molecule of NADH, which requires reoxidation to maintain the redox balance of the cell. In the absence of oxygen, this occurs by reduction of dihydroxyacetone phosphate to glycerol-3-phosphate and then to glycerol (Gancedo et al., 1968). Sodium carbonate or magnesium carbonate/hydroxide are commonly used to steer the alkali yeast process (Neuberg and Farber, 1917; Eoff et al., 1919; Schade and Farber, 1947).

The sulfite-steered yeast process was used for commercial production of glycerol during World War I in Germany. Six thousand tons of beet sugar (sucrose) were fermented by *S.*

Table 3
Some representative organisms used for glycerol production

| Strains | Substrate | Process/scale | Concentration of glycerol in broth (g/L) | Conversion efficiency (% consumed sugar) | Average productivity (g/(L day)) | Reference |
|---|-----------------|--|--|--|----------------------------------|---------------------------------|
| <i>Yeasts</i> | | | | | | |
| <i>S. cerevisiae</i> | Molasses | Batch sulfite/12 L | 55 | 25 | 11.5 | Cocking and Lilly, 1919 |
| | Glucose | Batch insoluble sulfite/2.5 L | 35 | 23 | 11.6 | Underkofler, 1954 |
| | Glucose | Continuous sulfite/pilot plant | 50 | 28 | 33 | Freeman and Donald, 1957a |
| | Molasses | Fed batch sulfite/vacuum, CO ₂ sparging | 80 | 25 | 30 | Kalle et al., 1985 |
| <i>C. magnoliae</i> I ₂ B ^a | Glucose | Batch alkali steered/12 L | 45 | 23 | 9 | Schade and Farber, 1947 |
| | Glucose | Aerobic batch process/shake flask | 79 ^b | 43 | 20 | Spencer and Shu, 1957 |
| <i>P. farinosa</i> | Glucose | Aerobic fedbatch/60 L | 170 | ND ^c | 17 | Peterson et al., 1958 |
| | Glucose | Aerobic fedbatch/1 L | 300 | ND | 75 | Vijaikishore and Karanth, 1986a |
| <i>C. glycerinogenes</i> | Glucose | Aerobic batch process/ | 110–130 | 52–63 | 28–32 | Zhuge and Fang, 1995 |
| | | From shakes flask to 50 000 L | | | | |
| <i>Bacteria</i> | | | | | | |
| <i>B. subtilis</i> | Glucose | Batch/shake flask | 14.7 | 29 | 2 | Neish et al., 1945 |
| <i>Algae</i> | | | | | | |
| <i>D. tertiolecta</i> | CO ₂ | Batch | 0.12 | ND | 0.066 | Ben-Amotz and Avron, 1981 |

^a Earlier called as *T. magnoliae* I₂B.

^b Total polyols.

^c Not determined or not relevant.

Cerevisiae monthly to yield 1000 tons of 'dynamite' glycerol (Bernhauer, 1957). The yeast was grown in a medium composed of 100 g/L beet sugar, 30 g/L Na₂SO₃ and salts. The anaerobic fermentation conducted in 1000 m³ fermentors was completed after 3 days at 30–35°C. At the end of fermentation, the broth contained about 20–30 g/L glycerol, 20–30 g/L ethanol and about 10 g/L acetaldehyde (Bernhauer, 1957). The maximum conversion efficiency of glycerol in this process reached about 20% of the metabolized sugar. It was also used to produce the glycerol in World War II in Germany, Japan, the former Soviet Union, Poland and Brazil. A group in Wisconsin (USA) also carried out a pilot plant study on the process to evaluate the economic potential of producing glycerol from sugar (Harris and Hajny, 1960). However, by 1960, production of glycerol by fermentation of sugar was found to be uneconomic when compared to chemical synthesis from petrochemical feedstocks (Freeman and Donald, 1957a,b,c). In China, the Chinese Academy of Science also carried out research on sulfite-steered yeast processes from 1967 to 1971. The *S. cerevisiae* strain 2-1190 was inoculated into broth containing 150–160 g/L sucrose in a 20-m³ fermentor and sodium sulfite were added by continuous feeding. Within 72 h, concentrations of 42.7 g/L glycerol and 37.0 g/L ethanol were obtained. The conversion efficiency of glycerol based on the amount of total sugar used was about 27%, but was regarded to be too low for commercial development.

Pilot plant studies on the alkali process have been conducted in a 10-m³ fermentor containing 165 g/L sugar. The fermentation required 5–7 days for completion and yielded 31 g/L glycerol and 67.5 g/L ethanol (Eoff et al., 1919). Subsequently, blackstrap molasses was used to produce glycerol in a 60-m³ fermentor, but the yield was never greater than 27% (Lawrie, 1928). The poor conversion of sugar to glycerol by the alkali process compared to the sulfite process resulted in this process not being commercialized.

Attempts to improve the alkali and sulfite processes by controlling aeration, by sparging with carbon dioxide or by applying a vacuum to the fermentor (Kalle et al., 1985; Kalle and Naik, 1987; Vikar and Panesar, 1987) have resulted in glycerol concentrations of up to 230 g/L and glycerol yields of up to 40% being achieved. The use of *S. cerevisiae* immobilized cells in a carbon dioxide-sparged fed-batch or continuous fermentor also improved the glycerol concentration and fermentation productivity but with only slight improvements in the glycerol yield (Bisping et al., 1989; Hecker et al., 1990).

Although these two processes of glycerol production are relatively simple, a number of technical problems have limited recent commercial application (Freeman and Donald, 1957a; Vijaikishore and Karanth, 1986b; Rehm, 1988; Agarwal, 1990). These include: (1) a relatively low glycerol yield as other by-products such as ethanol, acetaldehyde and acetic acid are also produced in significant amounts; (2) high amounts of sulfite or other steering agents are required during the fermentation; and (3) a low glycerol productivity rate and a low final glycerol concentration in the fermentation broth make the recovery of glycerol expensive and inefficient.

3.2. Production by osmotolerant yeasts

Nickerson and Carroll (1945) first noted the production of glycerol by osmotolerant yeasts. They discovered that significant glycerol production by an osmotolerant yeast strain,

Zygosaccharomyces acidifaciens, did not require a steering agent. This discovery stimulated a comprehensive investigation of osmotolerant or osmophilic yeasts in the hope that an organism producing glycerol with an attractive yield and without a need for a steering agent might be found. In addition to glycerol, osmotolerant yeasts were found to produce other polyols including erythritol, D-arabitol and mannitol (Spencer and Sallons, 1956). Since then, glycerol production by osmotolerant yeasts has been extensively investigated (see Onishi, 1963; Spencer and Spencer, 1978; Rehm, 1988; Agarwal, 1990 for earlier reviews).

Most osmotolerant yeast species considered for glycerol production belong to the genera *Candida*, *Debaryomyces*, *Hansenula*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, *Torulasporea* and *Zygosaccharomyces* (Onishi, 1963; Zhang et al., 1963; Zhuge, 1973; Kumar et al., 1989; Petrovska et al., 1999). The main advantages of osmotolerant yeasts for glycerol production compared to the processes based on alkali and sulfite are the following: (1) aerobic rather than anaerobic or oxygen-limited conditions are required for cell growth and fermentation; (2) no steering agents or osmotic solutes are needed; (3) considerably higher sugar concentrations can be used with an improved glycerol production rate and yield; and (4) much simpler process technology is used with less contamination.

Since initial studies have shown that 20% glycerol yields could be obtained from glucose by *Z. acidifaciens* (Nickerson and Carroll, 1945), other workers have attempted to improve the glycerol yields, rates of glycerol production and fermentation processes by osmotolerant yeasts. Glycerol yields as high as 40% have been obtained using a strain of *Candida magnoliae* (Hajny et al., 1960). Further improvements were attempted by cell recycling (Vijaikishore and Kranath, 1984), cell immobilization (Höötman et al., 1991) and continuous fermentation in fluidized bed reactors (Vijaikishore and Kranath, 1986a). None of these studies evidently led to a process that could be commercially exploited.

An intensive effort has been made in China since the 1970s to develop a glycerol process based on osmotolerant yeasts. Zhuge (1973) isolated a strain from among 5000 osmotolerant yeast isolates with considerable promise for glycerol production. This strain isolated from glazed fruit in Southern China was later identified as *C. glycerinogenes* (Wang et al., 1999a,b). In shake-flask culture experiments, the strain was found to convert 45% of total sugar to glycerol with a glycerol concentration of 85 g/L. Pilot plant experiments show that glycerol could be produced from a number of raw materials. For example, glucose obtained by enzymatic hydrolyses of sweet potato was fermented to 85 g/L glycerol with 41% conversion efficiency in a 3-m³ bioreactor (Zhuge, 1974). Subsequent fermentation of acid-hydrolysed corn starch in bioreactors varying between 0.5 and 2.5 m³ gave an average glycerol concentration of 100 g/L and 40% conversion efficiency (Zhuge, 1974). A typical fermentation time course for glycerol production is shown in Fig. 2. By using the genetically improved *C. glycerinogenes*, up to 130 g/L glycerol was produced with a conversion efficiency up to 63% and volumetric production rate up to 32 g/(L day) in a laboratory scale fermentor. Meanwhile, relatively low concentrations of lactate, acetaldehyde and ethanol were produced. No other polyols were found to accumulate. Conversion efficiency up to 58%, production rate up to 30 g/(L day) and glycerol concentrations between 110 and 120 g/L have been also obtained on commercial scale in an optimized aerobic fermentation process (Fig. 3; J. Zhuge, unpublished data).

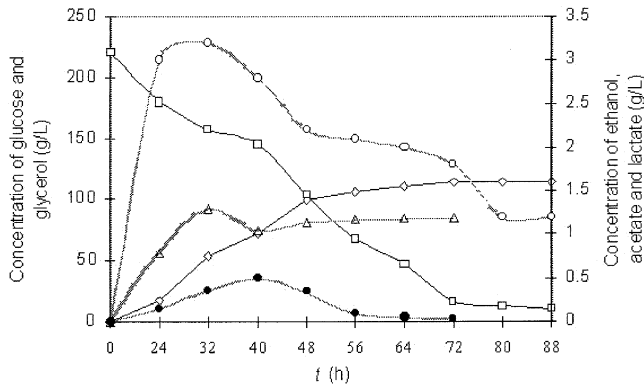


Fig. 2. A typical time course of glycerol production from glucose by *Candida glycerinogenes* at 31°C: □ glucose, ◇ glycerol, ○ ethanol, △ acetate, ● lactate. The concentration of glycerol in the broth reached 114 g/L after 72 h fermentation. The major byproducts were ethanol, acetate and lactate but the cells reutilized the ethanol and lactate. No other polyols were formed (J. Zhuge, unpublished data).

3.3. Production by bacteria

Glycerol production by bacteria has received scant attention and processes based only on *Lactobacillus lycopercisi* and *Bacillus subtilis* have been shown to have promise (Neish et al., 1947). For example, a *B. subtilis* strain yielded 29.5% glycerol in a glucose–yeast extract medium within 8 days (Neish et al., 1945). Since then, bacteria have not been seriously considered for glycerol production because of the slow fermentation rate and relatively low yields (Table 3). The recent demand for 1,3-propanediol as a feedstock for chemical synthesis of a polyester has led to the microbial synthesis route from glucose via glycerol by bacteria being considered (Biebl et al., 1999). The cloning of yeast and bacteria genes involved in glycerol metabolism into a variety of bacteria has resulted in a patented process based on a

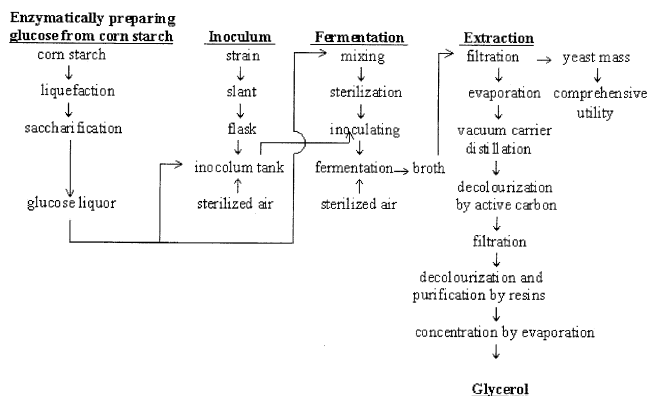


Fig. 3. Flow chart for glycerol production by *Candida glycerinogenes* from corn starch by aerobic cultivation in a 50 m³ airlift fermentor (Zhuge and Fang, 1994).

recombinant organism able to directly ferment glucose to 1,3-propanediol (Laffend et al., 1996). However, further improvements in the yields and productivities of the process are necessary before commercial development can be considered (Biebl et al., 1999).

3.4. Production by algae

The autotrophic production of glycerol using the green alga species *Dunaliella tertiolecta* and *Dunaliella bardawil* is an attractive possibility as CO₂ and light can be used as carbon and energy sources. In these algae, glycerol is accumulated intracellularly in response to increases in NaCl concentration (Ben-Amotz and Avron, 1981; Ben-Amotz, 1983). Extracellular glycerol concentrations up to 5 g/L have been reached in the hypersaline (4 M NaCl) medium of *D. tertiolecta* immobilized in calcium alginate (Grizeau and Navarro, 1986). The application of algae to glycerol production has apparently received no further attention.

4. Environmental regulation of glycerol synthesis

Environmental factors such as temperature, aeration, phosphate and nitrogen, and sugar concentration and osmotic stress have been found to effect the rate and yield of glycerol produced by yeasts. The optimum temperature for glycerol production by yeasts is apparently similar to the optimum growth temperature of the organism. For example, the highest levels of glycerol were produced by *S. cerevisiae*, *C. magnoliae* and various strains of *Z. rouxii* between 30°C and 35°C (Spencer and Shu, 1957) and by *C. glycerinogenes* between 29°C and 33°C (J. Zhuge, unpublished data). Both the glycerol yield and cell growth was greatly reduced at 40°C for *C. magnoliae* and at 35°C for *C. glycerinogenes*. Wine yeast strains of *S. cerevisiae*, however, have an optimum temperature between 20°C and 25°C for glycerol production (Gardner et al., 1993).

The degree of aeration has a marked effect on glycerol production in osmotolerant yeasts suggesting a requirement for optimized conditions (Spencer and Shu, 1957; Peterson et al., 1958; Hajny et al., 1960). For example, at low levels of aeration, more glucose is converted to ethanol and ethyl acetate by *C. glycerinogenes*, whereas glycerol production decreased significantly. Under highly aerated conditions, glucose utilization increased with cell biomass and more rapid growth, but the yield of ethanol and glycerol also decreased (Jin et al., 2000). The optimized dissolved oxygen concentration (DO) for *C. glycerinogenes* for glycerol formation was 1.34 mg/g dry weight and the oxygen consumption rate was about 16 mg/(g h) in a 5-L fermentor (Jin et al., 2000). However, the optimum DO for glycerol production is also affected by other process parameters such as culture temperature and components of medium especially concentration of phosphate necessitating optimization for each specific process (J. Zhuge, unpublished data). Compared to processes using osmotolerant yeasts, steered glycerol production by *S. cerevisiae* requires oxygen-restricted conditions. For example, sparging with carbon dioxide or applying a vacuum to the fermentor improved the glycerol concentration and fermentation productivity (Kalle et al., 1985; Bisping et al., 1989; Hecker et al., 1990).

The effect of nutrients on glycerol production has only been investigated to a limited extent. In one study on *C. glycerinogenes*, the optimum total phosphate concentration for glycerol production in a medium containing 230 g/L glucose was 65 mg/L (Zhuge, 1973, 1974). Furthermore, glycerol synthesis by *S. cerevisiae* may enhance phosphate cycling in the cell and, hence, would increase the glucose flux through glycolysis (Thevelein and Hohmann, 1995). The concentration of nitrogen in the form of urea has little effect on glycerol production (Peterson et al., 1958; Hajny et al., 1960; Zhuge, 1974). However, the nitrogen source in the medium of anaerobically grown *S. cerevisiae* has a significant impact on the glycerol yield (Omori et al., 1995; Albers et al., 1996). In ammonium-grown cultures, the glycerol yield is more than double that of cultures grown with an amino acid mixture as nitrogen source. As ammonium-grown cultures require de novo amino acid synthesis, the excess NADH formed is reoxidized via glycerol synthesis.

Increases in sugar concentration generally results in greater synthesis of glycerol by yeasts. The increase in glycerol concentration is due, in part, to the greater osmotic stress imposed on the organism (Vijaikishore and Karanth, 1986a; Andre et al., 1991; Albertyn et al., 1994). For example, the glycerol yield in continuous culture of *S. cerevisiae* was three- to fourfold greater at 0.971 a_w than at 0.994 a_w (Kenyon et al., 1986). In *C. glycerinogenes*, an increase in the concentration of glucose from 150 to 250 g/L significantly increased the glycerol concentration and that the optimal concentration of glucose was between 220 and 260 g/L (J. Zhuge, unpublished data). However, the consumption rate of glucose in batch culture decreased. This results in a slower rate of glycerol production and, hence, decreasing the water activity of the medium does not necessarily improve the overall production efficiency (Onishi, 1957; J. Zhuge, unpublished data).

5. Recovery of glycerol

The recovery of glycerol from fermentation broth has been a major factor limiting the commercial application of the biological route of glycerol production. The high concentration of dissolved solids in the fermentation broth, the low glycerol concentrations in the sulfite-steered process and the high glycerol boiling point are difficulties that have hindered development of an efficient glycerol recovery process (Underkofler, 1954; Agarwal, 1990). Vacuum distillation after ethanol distillation and precipitation of salts such as sulfite, sulfates and phosphates is a simple process for glycerol recovery but results in approximately 50% of glycerol not being recovered (Underkofler, 1954; Weixl-Hofmann and von Lacroix, 1962; Rehm, 1988). An alternative recovery process linked to continuous glycerol production by *S. cerevisiae* from molasses has been developed using ion exchange resins (Harris and Hajny, 1960). In this recovery process, a combination of ion exclusion and ion exchange chromatography removed over 95% of the ionic impurities and 92% of the nonionic impurities. However, a relatively complex pretreatment of the fermentation broth is required to prevent inactivation of the ion exchange resin.

The cell debris and other dissolved organic substances in the fermentation broth form a solid mass that traps the glycerol and retards evaporation during distillation at 160–180°C. A solution to this problem of inefficient distillation was found by removing the cell debris and

dissolved solids by filtration followed by adding an inorganic inert material to the broth. More than 90% glycerol could be recovered by distillation from the broth containing the carrier using this patented technique called carrier-distillation (Zhuge and Liu, 1990). If combined with an ion-exchange chromatography for purification, 1 ton of medical grade glycerol can be produced from 3.1 tons of commercial starch (Fig. 3; J. Zhuge, unpublished data). Furthermore, this method can also be used to recover glycerol as a byproduct of ethanol fermentation or other fermentations.

6. Genetic improvement of glycerol synthesis

Mutagenesis, strain breeding or overexpression of the genes associated with glycerol formation has been attempted in efforts to improve glycerol synthesis by microorganisms. Almost all of the attempts are based on channeling the glycolytic flux towards glycerol formation and on decreasing the activities of the pathways for dissimilation of glycerol.

Triose phosphate isomerase is a key enzyme in the glycolysis that directs dihydroxyacetone phosphate to glyceraldehyde-3-phosphate after the split of fructose-1,6-bisphosphate. When the triose phosphate isomerase gene (*TPI*) of *S. cerevisiae* is deleted, the mutant was able to attain a high glycerol yield from glucose (80–90% of the theoretical yield) and glycerol productivity (1.5 g/(L h)) without the need for a steering agent (Compagno et al., 1996). However, the mutant grows poorly due to an energy deficiency and showed genetic instability on glucose.

The NAD⁺-dependent glycerol-3-phosphate dehydrogenase is a key enzyme for glycerol formation in *S. cerevisiae* and many other yeast strains (Albertyn et al., 1994; Wang et al., 1994; Ohmiya et al., 1995; Wang and Zhuge, 1999a) and the overexpression of the *GPD1* gene in yeast increases glycerol production. The glycerol yield in a strain of *S. cerevisiae* exhibiting 20-fold increased Gpd1p activity resulting from overexpression of *GPD1* gene was 6.5 times that of the wild type (Nevoigt and Stahl, 1996). Overexpression or disruption of *GPD1* also modulate glycerol and ethanol yields during alcoholic fermentation in *S. cerevisiae* (Nevoigt and Stahl, 1996; Michnick et al., 1997). Wine yeast strains with modulated *GPD1* expression have been constructed and characterized during fermentation on glucose-rich medium. Mutants with *gpd1Δ* exhibited a 50% decrease in glycerol production and increased ethanol yield. On the other hand, overexpression of *GPD1* in strains resulted in a substantial increase in glycerol production at the expense of ethanol in broth containing 200 g/L glucose. Coupled to the higher glycerol levels were significant increases in the accumulation of by-products such as pyruvate, acetate, acetoin, 2,3-butanediol and succinate (Michnick et al., 1997; Roustan et al., 1999). All these genetically improved yeast strains produce less than 20 g/L glycerol from 200 g/L glucose when cultivated under conditions similar to those used in wine production (Nevoigt and Stahl, 1996; Michnick et al., 1997; Roustan et al., 1999). No reports have yet appeared on the ability of recombinant osmotolerant yeasts overexpressing *GPD1* to produce elevated levels of glycerol. However, as the glycerol metabolism in

yeast becomes better understood, more tools would become available to genetically manipulate yeasts to overproduce glycerol.

S. cerevisiae mutants resistant to amino acid analogues have improved production of amino acids such as leucine and phenylalanine and this might cause a decrease in alcohol dehydrogenase activity thereby improving glycerol productivity (Omori et al., 1995). Knocking out genes involved in the synthesis of arabitol in *C. glycerinogenes* resulted in increased glycerol production from 85 g/L to more than 100 g/L (J. Zhuge, unpublished data).

The breeding of *S. cerevisiae* strains to overproduce glycerol has only received attention with the purpose of improving wine properties (Eustace and Thornton, 1987; Prior et al., 1999). Studies have shown that up to three-fold increases in glycerol production can be achieved crossing selected strains. However, only glycerol levels of 15 g/L have been reached, and this is coupled with elevated levels of other metabolites suggesting this approach is unlikely to be used to breed a yeast strain for commercial glycerol production.

7. Conclusions and future prospects

There has been a significant improvement in the microbial production of glycerol by strain manipulation and selection when compared with the processes that were used in the first half of the twentieth century. In China, glycerol fermentation technology has been developed to a stage that commercial production occurs and approximately 10 000 tons/year is currently produced by microbial fermentation which supplies more than 12% of the country's needs. The success of this commercial process was largely achieved by isolation of yeasts able to ferment glucose to glycerol in high concentrations, yields and productivities and the development of an efficient glycerol recovery technology. It is unknown whether this process would be economically viable in other countries where traditional processes based on glycerol recovery from fats and other agricultural products are dominant. Furthermore, improvements in the glycerol yields and productivities of yeast strains would help to improve the economics of the glycerol fermentation processes. The understanding of glycerol metabolism in yeast such as *S. cerevisiae* has made significant progress over the past ten years but up to now, the knowledge has only been applied to the manipulation of wine yeasts. The challenge for the biotechnologist will be to apply the fundamental knowledge on glycerol metabolism to manipulate yeast strains appropriate for economic glycerol production by microbial fermentation.

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