1 Introduction

1.1. Violacein and deoxyviolacein

Violacein and deoxyviolacein are secondary metabolites produced by a diverse group of microorganisms. The pigments were firstly described in 1882 when the compounds were extracted with ethanol and the spectrophotometric properties of the obtained solutions were analyzed (Boisbaudran, 1882). These molecules present blue/violet color, which facilitates the isolation of natural producer strains for further research and enable its use as a bio-dye that provides good color tone and stability (Shirata et al., 2000). Their biological role was studied using bacterial strains isolated from the environment (Matz et al., 2008). In order to analyze growth and survival rates of bacterivorous nanoflagellates, isolates of the violacein and deoxyviolacein producer strains *Janthinobacterium lividum* and *Chromobacterium violaceum* were used to feed these organisms. It was demonstrated that the uptake of less that than three pigmented bacterial cells caused cell death in less than 20 min to these microorganisms. The results of the study suggest that the production of the pigments is related to a defense mechanism against predation. In another study, it has been determined that the presence of *J. lividum* on the skin of the salamander species *Plethodon cinereus* increased the survival rates of this amphibian when exposed to the fungus *Batrachochytrium dendrobatidis*, the causative agent of the disease chytridiomycosis one of the causes for the decline in amphibian populations (Becker et al., 2009b). A threshold concentration of about 18 µM violacein prevented mortality caused by *B. dendrobatidis*. Due its biological properties, violacein and deoxyviolacein draw attention of researchers as possible candidate compounds for the development of drugs for cancer treatment among other biotechnological and medical applications.

1.1.1. Biotechnological and therapeutic potential of violacein and deoxyviolacein

Violacein and deoxyviolacein extracted from *J. lividum* are effective against *Rosellinia necatrix*, which is responsible for white root rot of mulberry. The use of

violacein and deoxyviolacein against this phytopathogenic fungi, demonstrated the fungicide potential of these compounds (Shirata et al., 2000).

It was observed that a mixture of violacein and deoxyviolacein is effective against many gram-positive strains of microorganisms and to some extent against gram-negative. They are active against bacteria such as *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* in concentrations ranging from 10-15 mg L^{-1}. Depending on the concentration tested, the pigment mixture was able to cause growth inhibition to cell death (Nakamura et al., 2003; Nakamura et al., 2002). Particularly in the case of *S. aureus*, which is an opportunistic pathogen frequently involved in infections, alternative antibiotics for human treatment are in demand due to the increasing development of resistance against the current medicines available. The treatment of *S. aureus* human infections is done using antibiotics, such as methicillin or vancomycin. Strains resistant to methicillin have been described that could be treated with vancomycin; however, strains of *S. aureus* resistant to methicillin and vancomycin up to serum concentrations of 9.4 mg L^{-1} (Howe et al., 1998) were isolated, bringing concern to the medical community due to the lack of effective antibiotic alternatives for treatment (Sieradzki et al., 1999). Violacein loaded nanoparticles presented two to five times reduction of the minimal inhibitory concentration compared to the free form of the pigment against strains of *S. aureus* (Martins et al., 2011).

Tuberculosis is a world-wide disease caused by *Mycobacterium tuberculosis*. In 2011, 8.7 million new cases were estimated and 1.4 million died, 430,000 of which were also infected with HIV (WHO, 2012a). Conventional medication for treatment of tuberculosis is becoming ineffective, as occurrence of multidrug-resistant variants of *Mycobacterium tuberculosis* is increasing and reached 60,000 cases world-wide in 2011 (WHO, 2012a). Violacein exhibited activity against *Mycobacterium tuberculosis* (Mojib et al., 2010). The minimum inhibitory concentration of violacein extracted from *Janthinobacterium* sp. was 34.4 mg L^{-1} for the virulent strain $H_{37}Rv$ demonstrating the potential of violacein for the treatment of tuberculosis.

Experiments conducted to test the effect of violacein and deoxyviolacein mixtures in concentrations above 50 mg L^{-1} presented no growth inhibition or cell death against *Escherichia coli* HB 101 (Nakamura et al., 2003). Even when

violacein was loaded in polymeric poly-(D,L-lactide-co-glycolide) nanoparticles to improve solubility in water, no significant antibacterial activity occurred against *E. coli* in concentrations up to 68.5 mg L^{-1} (Martins et al., 2011). The ability of *E. coli* to cope with high violacein and deoxyviolacein concentrations is an important parameter regarding the potential utilization of this microorganism for production purposes, as further discussed below.

Poliomyelitis is a highly infectious viral disease that can cause paralysis (Bonnet and Dutta, 2008) and affects mainly children under five years of age (WHO, 2012b). This disease can be prevented by vaccination; however, there is no specific treatment available (Bonnet and Dutta, 2008). In addition, the World Health Organization estimates that failure to eradicate polio in countries like Afghanistan, Nigeria, and Pakistan could make the disease spread all over the world within 10 years (WHO, 2012b). Experiments performed using extracts containing violacein and deoxyviolacein showed that these compounds can prevent human cell infection by the poliomyelitis virus (May et al., 1991). These extracts presented also activity against Herpes simplex virus cell infection (May et al., 1991).

Violacein is also active against *Leishmania amazonensis* (Leon et al., 2001) and *Trypanosoma cruzi* (Durán et al., 1994) the causative agents of Leishmaniasis and Chagas diseases, respectively. These diseases are members of the group of neglected diseases (Trouiller et al., 2002) or more specifically, diseases for which no investments are made in research due to low profitability of the market formed by the low-income people that are affected by them.

Leukemia is a type of cancer which affects the blood and the bone marrow. In the year 2000, it was estimated that this disease affected 255,932 people causing 209,328 deaths world-wide (Mathers et al., 2001). Violacein obtained from *C. violaceum* presented potential as a drug for leukemia treatment (da Silva Melo et al., 2000). It was effective against HL60 leukemic cells (Ferreira et al., 2004) an established model for studying myeloid leukemia. The mechanism by which violacein induced apoptosis in HL60 cells is related to the activation of the tumor necrosis factor (TNF) receptor signaling (Ferreira et al., 2004). In order to improve the efficiency of violacein against HL60 cells, the use of nanoparticles was tested. The fact that tumor cells have a higher ascorbic acid uptake rate motivated the use of violacein-loaded nanoparticles capped with ascorbic acid as a way to improve

the efficiency against tumor cells. This system was 2-fold more effective than free violacein as antitumoral drug (Martins et al., 2010). In another study (Gimenez et al., 2005), violacein complexed by cyclodextrin-thiol-protected gold nanoparticles presented improved water solubility as compared to free violacein. These nanoparticles presented reduced toxicity activity against normal V79 cells while maintaining the activity against myeloid leukemia HL60 cells. These results show that nanoparticles are an important tool for the targeted delivery of antitumoral compounds, such as violacein and deoxyviolacein, to cancer cells in order to reduce the side effects of chemotherapy.

Colorectal cancer is the fourth most common cancer type (Boyle and Langman, 2000) and the third cause of cancer death (Parkin, 2001). It is estimated that colorectal cancer is responsible for 394,000 deaths annually affecting mainly Europe, North America, and Australasia. In the European Union it is the second cause of men death among the cancer types (Boyle and Langman, 2000). The chemotherapy employed for the treatment of colorectal cancer is done using 5-fluorouracil (Heidelberger et al., 1957), which is the most widely used drug for this type of cancer (Gill et al., 2003), in combination with other compounds such as leucovorin (Machover, 1997). In a phase III study, it was demonstrated that patients treated with either a combination of 5-fluorouracil and leucovorin or 5-fluorouracil, leucovorin and oxaliplatin presented 16 and 53% objective response, respectively (Giacchetti et al., 2000). The objective response was determined by extramural reviews of computed tomography scans. It has been demonstrated that violacein is an effective cytotoxic compound against colorectal cancer cells (Kodach et al., 2006). Violacein could induce apoptosis in colorectal cancer cells by blocking the phosphatidylinositol-3-kinase/Akt (Vivanco and Sawyers, 2002) activation and could increase the chemosensitivity of the cells to 5-fluorouracil when used in combination with this compound. The mechanism by which violacein induces death in the Caco-2 cell line, which is a model for colorectal cancer studies, involves the formation of reactive oxygen species that ultimately culminate in tumor apoptosis (De Carvalho et al., 2006). These results demonstrate the potential application of violacein as chemotherapeutic agent for colorectal cancer treatment. Moreover, violacein and deoxyviolacein were also able to inhibit hepatocellular carcinoma cell proliferation (Jiang et al., 2012).

In addition to *in vitro* studies on the antitumoral properties of violacein, *in vivo* studies were also performed using Ehrlich ascites tumor cells (EAT) as model (Bromberg et al., 2010). Experiments demonstrated that EAT cells were two times more sensitive to violacein than the normal cells. The experiments conducted using mice injected with EAT cells intraperitoneally showed that violacein doses ranging from 0.1 to 1 μg kg^{-1} significantly reduced tumor growth. Moreover, daily violacein doses of 1000 μg kg^{-1} did not cause toxicity to kidneys and liver when injected intraperitoneally.

Antiulcerogenic activity of violacein (Durán et al., 2003) has been demonstrated in experiments using mice as model. Orally administered violacein significantly reduced gastric lessons. Further improvements of the protective effect of violacein were possible by using violacein beta-cyclodextrin (betaCD) inclusion complexes. In this case, reduction of gastric damage of about 85% was possible using violacein loaded betaCD inclusion complexes with 1:2 ratio.

1.2. E. coli *as industrial production host*

E. coli is one of the most used hosts for production of recombinant compounds in industry, due to its well known physiology, availability of tools for genetic manipulation, ease of scale-up, low-cost media and rapid growth (Huang et al., 2012). Specifically, the strain K-12 has been established as standard and received guidelines for safety regarding therapeutics production. One third of the recombinant therapeutics approved by the US Food and Drug Administration and the European Medicines Agency are produced in *E. coli* (Huang et al., 2012). Some examples of approved recombinant therapeutics produced in *E. coli* are Ranibizumab (Lucentis), Somatropin (Accretropin), Certolizumab pegol (Cimzia), PEG interferon alfa-2b (PegIntron), Romiplostim (Nplate), and Interferon beta 1b (Extavia) Pegloticase (Krystexxa). Important advancements have been made in recent years towards the production of complex molecules that require disulfide bond formation and glycosylation. The functional expression of the N-linked glycosylation pathway of *Campylobacter jejuni* in *E. coli* opened up the possibility to generate compounds that require this important type of modification, frequently necessary in drugs for treatment of diseases and for research (Wacker et al.,

2002). In addition to therapeutics production, *E. coli* is also an import host for production of a diverse group of chemicals for different purposes.

Currently, an increasing tendency is observed regarding the use renewable resources for production of commodities and fine chemicals (Bozell and Petersen, 2010). Due to concerns about negative environmental impacts of fossil based resources, as well as limitations in the availability or political instability in the producing countries, biotechnological based production appeared as a potential alternative to overcome these issues. Advancements in research along the years are making the economical feasibility of many biotechnological processes approach a state in which competition with the petrochemical industry can be considered. An indication of this trend is the growing global value of biotechnology industry, which increased from 54 billion dollars in 1999 to about 500 billion dollars in 2011 (Bruschi et al., 2011). An example of chemical produced in industrial scale by *E. coli* is 1,3-propanediol (Laffend et al., 1997), produced since 2006 with a 45,000 tonne/year capacity (Burk, 2010). Another promising compounds, but not limited to, are fuels (1-propanol and 1-butanol), amino acids (L-threonine, L-valine, L-phenylalanine, L-tryptophan, and L-tyrosine), succinic acid, xylitol, and 1,4-butanediol (Burk, 2010; Chen et al., 2013).

1.3. *Physiology and metabolism of* E. coli

In this section, a brief overview of the main pathways of the metabolism of *E. coli* will be given. Relevant aspects of the metabolism, e.g., transport and catabolism of glucose, glycerol, and L-arabinose, as well as the mechanisms of control of gene expression, will be presented. Finally, the L-tryptophan pathway will be covered concerning the relevant factors related to the improvement of the metabolic fluxes for biosynthesis of this fundamental amino acid for violacein and deoxyviolacein production. If not stated otherwise, the information presented in this section was based on the comprehensive works of (Keseler et al., 2005; Neidhardt et al., 1996). A simplified schematic presenting the transport systems of substrates, the central metabolism and biosynthesis of L-tryptophan is presented in Figure 1.

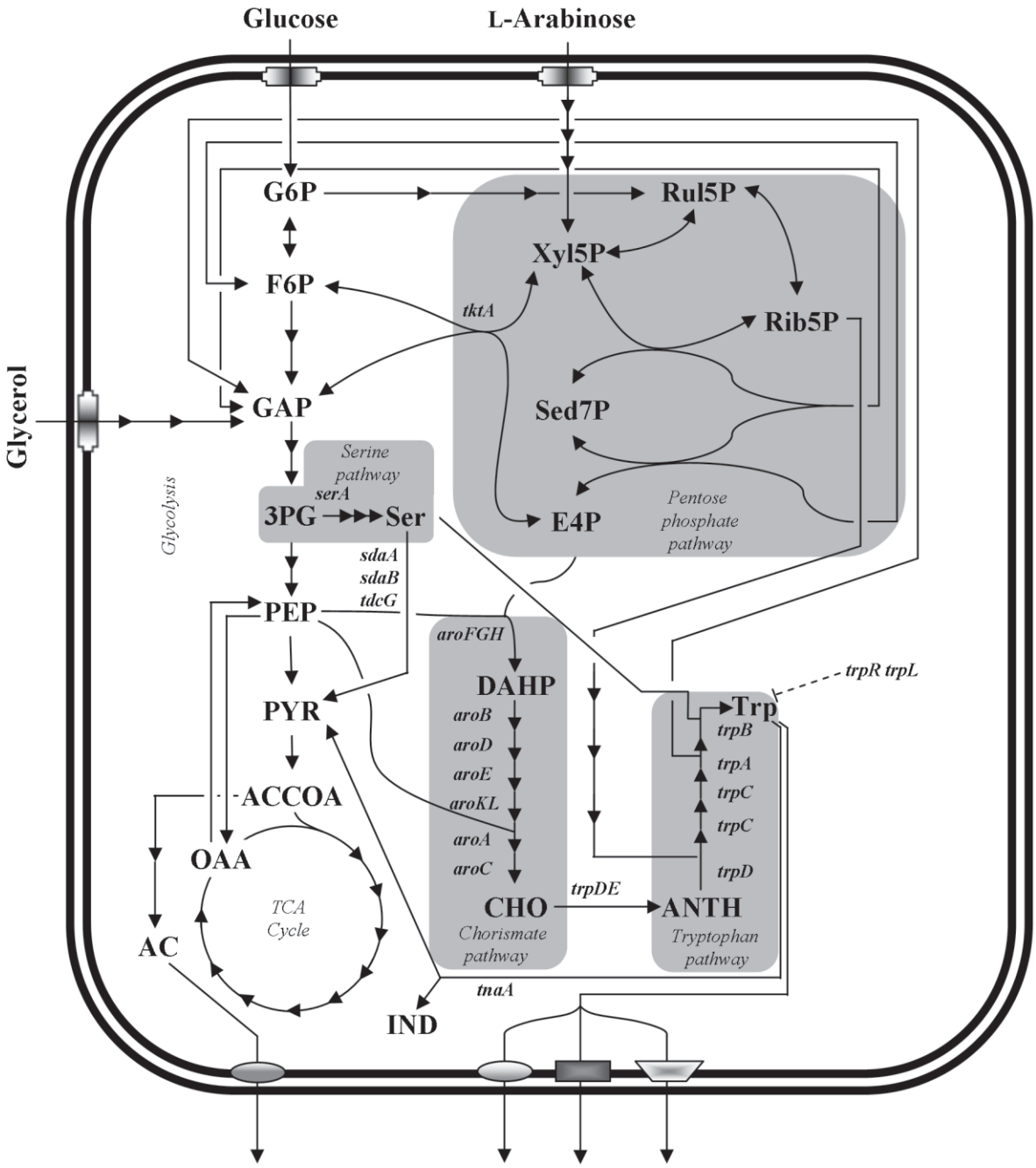

Figure 1. Central metabolism of *E. coli* and L-tryptophan pathway. Compound names: G6P (glucose 6-phosphate), F6P (fructose 6-phosphate), GAP (glyceraldehyde 3-phosphate), 3PG (3-phosphoglycerate), PEP (phosphoenolpyruvate), PYR (pyruvate), ACCOA (acetyl-CoA), OAA (oxaloacetate), IND (indole), Rul5P (ribulose 5-phosphate), Xyl5P (xylulose 5-phosphate), Rib5P (ribose 5-phosphate), Sed7P (sedoheptulose 7-phosphate), E4P (erythrose 4-phosphate), Ser (L-serine), DAHP (3-deoxy-D-arabino-heptulosonate-7-phosphate), CHO (chorismate), ANTH (anthranilate), Trp (L-tryptophan), IPA (indole-3-pyruvic acid imine), PDVA (protodeoxyviolaceinic acid), and AC (acetate).

1.3.1. Transport and metabolism of glucose, L-arabinose and glycerol

Glucose is one of the main carbon sources utilized by *E. coli*. This microorganism is able to metabolize glucose, in addition to some salts, as energy and carbon source to synthesize the other compounds required for growth. Similarly, L-arabinose and glycerol can also serve this purpose. The catabolism of glucose initiates with its transport into the cell. The most used glucose transporter in *E. coli* is the phosphoenolpyruvate phosphotransferase system (PTS). In addition to glucose, the PTS is responsible for the transport of different compounds into the cell, such as mannitol and mannose. Some components of the PTS have a general purpose, while other proteins involved exhibit specificity for the target compound to be transported. The driving force required for this type of transport to take place is derived from phosphoenolpyruvate (PEP). The phosphate of this molecule is ultimately transferred to the respective sugar, during transport from the periplasm into the cytoplasm, releasing pyruvate as byproduct. The PTS responsible for the uptake of glucose is composed of free and membrane-bounded proteins. Enzyme I (EI) and the histidine protein (HPr) encoded by the genes *ptsI* and *ptsH*, respectively; are the free cytoplasmic general-use component part of the system. The sugar specific EII component has also a free and a membrane-bounded part. In the case of the glucose transport, these components are called IIA^{Glc} or Crr and $IICB^{Glc}$ or PtsG encoded by the genes *crr* and *ptsG*, respectively. The transfer of the phosphate group of PEP to glucose occurs in following order: PEP → P-EI → P-HPr → P-$EIIA^{Glc}$ → P-$EIICB^{Glc}$ → glucose-6-P. The general proteins encoded by the genes *ptsH*, *ptsI*, and *crr* of the glucose PTS are clustered in an operon. This operon is constitutively expressed at a basal level, but the level of gene expression is increased upon growth on PTS substrates. The *ptsG* gene is inducible by glucose in *E. coli* K-12. One of the possible explanations for the fact that the *crr* gene is expressed together with the general components EI and HPr can be the role that this protein plays on the transport of other compounds, such as trehalose and sucrose. In addition to glucose transport, the PTS has other important functions in *E. coli*. In a process called inducer exclusion, the $EIIA^{Glc}$ component interacts with proteins related to the metabolism of other carbon sources, e.g., glycerol and lactose,

inhibiting their activity and, consequently, the use of these compounds for growth. This process takes place when the cell is using a PTS carbohydrate as carbon source. Only if some of the above mentioned carbon sources is present that the $EIIA^{Glc}$ protein acts as inhibitor. This occurs because the non-phosphorylated form of $EIIA^{Glc}$ is able to bind and inhibit the activity of different proteins related to the catabolism of alternative carbon sources that are available during the metabolism of glucose. For instance, in the presence of glucose and glycerol, *E. coli* will first transport glucose using the PTS. During the glucose transport, the predominant form of the $EIIA^{Glc}$ protein will be non-phosphorylated, as the phosphates are now being transferred to the transported glucose. As glycerol is also present, the $EIIA^{Glc}$ protein is able to bind to the enzyme glycerol kinase, thereby inhibiting the phosphorylation of glycerol to glycerol-3-phosphate, which is the first intermediate metabolite of the glycerol catabolism. Another import role played by the PTS is the regulation of the adenylate cyclase activity. This enzyme is responsible for the biosynthesis of cyclic AMP (cAMP), an important signaling molecule. The interaction of cAMP with the transcriptional dual regulator CRP (cAMP receptor protein) yields the cAMP-CRP complex which acts globally in the control of transcription initiation in *E. coli*. The phosphorylated form of the $EIIA^{Glc}$ protein stimulates the hydrolysis of ATP by the adenylate cyclase for cAMP production. Therefore, as the concentration of phosphorylated $EIIA^{Glc}$ is low during glucose catabolism, adenylate cyclase activity will also be reduced; on the other hand, it is increased during catabolism of non-PTS compounds. This is the basis of the regulation mechanism known as catabolite repression. An example of carbon source subject to catabolite repression regulation is L-arabinose, as further discussed below. After glucose is transported into the cell with concomitant phosphorylation to glucose-6-phosphate, this metabolite can be promptly catabolized as carbon and energy source by the pathways of the central metabolism.

The transport of L-arabinose, contrary to glucose, does not rely directly on PTS. There are two transport systems for L-arabinose in *E. coli*, a system with high-affinity and another with low-affinity, which are encoded by the *araFGH* and *araE* genes, respectively. L-Arabinose transport through the *araFGH* system is driven by ATP hydrolysis, while the *araE* system consists of a symporter that relies on the electrochemical gradient of protons for the transport of L-arabinose through

the cell membrane. The *araF* gene is responsible for the production of a periplasmic binding protein. The *araG* and *araH* genes encode proteins responsible for the ATP binding and the membrane attachment, respectively. The regulation of gene expression in both systems is controlled by L-arabinose, AraC protein, CRP, and cAMP. When L-arabinose is present, the AraC protein changes its conformation to act as inducer of gene expression. This form of AraC interacts with the cAMP-CRP complex and the RNA polymerase, thereby inducing the expression of the *araFGH* and *araE* genes. Conversely, in absence of L-arabinose, the AraC protein acts as repressor of gene expression. This is a useful characteristic, as it allows the genes under control to be tight repressed or induced, fact that can play a major role regarding stability of heterologous gene expression in *E. coli*. As mentioned above, availability of the cAMP-CRP complex is required for induction of gene expression by the AraC protein. Here is where the catabolism of L-arabinose is affected by catabolic repression. If *E. coli* is metabolizing a PTS compound, the phosphorylation level of the protein $EIIA^{Glc}$ will be low, as the level of the EI protein will also be reduced due to the phosphate demand required by the substrate being transported. Due to the reversibility in the phosphorylation reactions in the PTS, the concentration of the $P\text{-}EIIA^{Glc}$ will decrease, thus reducing the activity of adenylate cyclase and, consequently, the intracellular concentration of cAMP. At this moment, the PTS can indirectly affect, provided that a PTS substrate is available, the transport of L-arabinose by repressing *araFGH* and *araE* expression. After transport into the cell, L-arabinose undergoes a series of modifications before is can be catabolized by the central metabolism of *E. coli*. Once L-arabinose is available in cytoplasm, it is metabolized by the enzymes L-arabinose isomerase, L-ribulokinase, and L-ribulose 5-phosphate 4-epimerase of the *araBAD* operon. Similarly, the regulation of gene expression in this operon is subject to control by the AraC protein (Figure 2). In the first reaction, catalyzed by the enzyme L-arabinose isomerase, L-arabinose is converted to L-ribulose. This compound is phosphorylated by L-ribulokinase to yield L-ribulose 5-phosphate, which is finally converted to D-xylulose 5-phosphate and metabolized in central metabolism.

Glycerol transport in *E. coli* occurs through a protein channel encoded by the *glpF* gene. This transport is mediated by facilitated diffusion, so that this carbohydrate can be moved in each direction through the cell membrane. Once