# PURIFICATION AND CHARACTERIZATION OF PHOSPHOFRUCTOKINASE (PFK) ENZYME FROM ANTARCTIC YEAST, *LEUCOSPORIDIUM ANTARCTICUM*

**by**

**SHANMUGAPRIYA A/P PERUMAL**

**Thesis submitted in fulfillment of the requirements for the Degree of Master of Science**

**2008**

## ACKNOWLEDGEMENTS

I wish to express gratitude to my supervisor, Dr. Rashidah Abdul Rahim for her guidance, advices, wisdom and perseverance through difficult times. Her support made this project progress without much obstacle and beneficial to myself. I would also like to express my appreciation to my co-supervisor, Professor Mohd Nazalan Mohd Najimudin for his helps and attentions throughout the research period.

I would also like to pay my heartiest gratitude to Mr. Hasni for his valuable advices, suggestions, assistances, and scientific discussions of the project. Also thank you to my fellow students and friends especially Sabrina, and all other members of the Lab 414 and 409.

Appreciations are also expressed to the staff of the School of Biological Sciences, USM, especially Electron Microscopy Unit, Biotechnology Teaching Laboratory, and Microbiology Teaching Laboratory for providing facilities and assistances during the research being done.

## TABLE OF CONTENTS

**CHAPTER 4: RESULTS AND DISCUSSION**

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF PLATES

## LIST OF APPENDICES

## LIST OF SYMBOLS

| | |
|---|---|
| % | percent |
| $^{o}C$ | Degree Celsius |
| K | kilo |
| m | milli |
| μ | micro |
| α | alfa |
| β | beta |
| $V_t$ | Total gel bed volume |
| $V_o$ | Void volume |
| $V_e$ | Elution volume |

## LIST OF ABBREVIATION

| | |
|---|---|
| APS | Ammonium persulphate |
| ATP | Adenosine triphosphate |
| BSA | Bovine Serum Albumin |
| DNase | Deoxyribonuclease |
| DTT | Dithiothreitol |
| EDTA | Ethylene diamine tetraacetic acid |
| G3PDH | Glyceraldehyde 3- Posphodehydrogenase |
| HCl | Hydrochloric acid |
| hr | Hour |
| kDa | kilo Dalton |
| Mg | Magnesium |
| M | Molar |
| min | minute |
| mM | Millimolar |
| NaOH | Natrium Hydroxide |
| NADH | Nicotinamide adenine dinucleotide |
| nm | nanometer |
| OD | Optical density |
| PEP | Phosphoenolpyruvate |
| PPi | Pyrophosphate |
| pI | Isoelectric point |
| rpm | revolution per minute |
| SDS | Sodium dodecyl sulphate |

| | |
|---|---|
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| Sec | second |
| TEMED | Tetramethylethylenediamine |
| TPI | Triphosphate Isomerase |
| U | Unit |
| UV | Ultraviolet |
| V | Volt |
| v/v | volume/volume |
| W | Watt |
| w/v | weight/volume |
| w/w | weight/weight |

**PENULENAN DAN PENCIRIAN ENZIM FOSFOFRUKTOKINASE (PFK) DARI YIS ANTARTIKA, *LEUCOSPORIDIUM ANTARCTICUM***

## ABSTRAK

*Leucosporidium antarcticum* telah dipencilkan daripada sampel air dari Stesen Davis di Antartika dan menunjukkan potensi untuk menghasilkan enzim intrasel fosfofruktokinase (PFK) (ATP-PFK; EC 2.7.1.11) pada suhu 5 $^{o}$C di dalam medium kompleks. Sel pencilan yis ini berbentuk ovoid serta mempamerkan ciri reproduksi aseks dengan bertunas. Sel yis *L.antarcticum* didapati menghasilkan aktiviti enzim PFK intrasel di akhir fasa pertumbuhan eksponen (sebanyak 32 x $10^{8}$ CFU/mL) selepas 15 hari pengkulturan pada suhu 5$^{o}$C. Pengoptimuman parameter fizikal seperti suhu pada 5 $^{o}$C, kelajuan agitasi pada 150 rpm dengan saiz inokulum sebanyak 8% (v/v) (126 x $10^{7}$ CFU/mL) serta medium permulaan yang mempunyai pH 7.0 didapati meningkatkan penghasilan PFK dengan signifikan. Enzim ATP-PFK telah ditulenkan melalui kromatografi penurasan gel (ultrafiltrasi), kromatografi afiniti dan kromatografi penukaran anion. Penulenan ATP-PFK dari *L. antarcticum* menghasilkan 2.85 mg enzim PFK tulen dengan aktiviti spesifik sebanyak 5.92 mU/mg dan hasilan 7% serta 1.3 kali penulenan. Aktiviti enzim ATP-PFK oleh *L.antarcticum* telah ditentukan dengan kaedah asai spektrofotometri yang berdasarkan pada pengoksidaan NADH pada penyerapan 340 nm selama 5 min pada suhu 5 $^{o}$C. Enzim PFK yang tulen didapati homogenus pada gel poliakrilamida SDS dengan anggaran berat molekul subunit sebanyak 100 kDa pada SDS-PAGE. Kromatografi penurasan gel menunjukkan enzim tulen PFK berbentuk homodimer yang mempunyai berat molekul asli sebanyak 200 kDa. PFK tulen didapati mempunyai suhu optimum sebanyak 5 $^{o}$C serta pH optimum sebanyak

7.0. Ion $Mn^{2+}$, $Co^{2+}$ dan $K^{+}$ (25 mM masing-masing) didapati meningkatkan aktiviti PFK manakala $Cu^{2+}$, $Ca^{2+}$, $Fe^{2+}$, $Zn^{2+}$, and $Ni^{2+}$ merencatkan aktiviti PFK. *L.antarcticum* didapati mempunyai enzim PFK yang spesifik pada substrat F-6-P sebagai penerima fosforil. Kespesifikan nukleotida oleh PFK tulen membuktikan enzim PFK ini merupakan enzim ATP-PFK. Lengkuk kinetik menunjukkan ia mempunyai $K_m$ sebanyak 41.67 mM bagi F-6-P dan 52.63 mM bagi ATP. Manakala, $V_{max}$ bagi F-6-P dan ATP masing-masing adalah sebanyak 13.77 mU/mL dan 12.12 mU/mL.

**PURIFICATION AND CHARACTERIZATION OF PHOSPHOFRUCTOKINASE (PFK) ENZYME FROM ANTARCTIC YEAST, *LEUCOSPORIDIUM ANTARCTICUM***

## ABSTRACT

*L.antarcticum* was isolated from the freshwater sample collected from Davis Station in Antarctic and was potential to produce intracellular phosphofructokinase (PFK) (ATP-PFK; EC 2.7.1.11) enzyme at 5 $^{o}$C in the complex medium. The cells of the isolate were ovoid in shape and showed asexual reproduction by budding. *L.antarcticum* produces intracellular PFK activity in the late exponential growth phase (about 32x10$^{8}$ CFU/mL) after 15 days of incubation at 5 $^{o}$C. The optimization of physical parameters such as temperature of 5 $^{o}$C, agitation speed of 150 rpm with inoculum size of 8% (v/v) (126 x 10$^{7}$ CFU/mL) and initial pH medium of 7.0 were found to enhance the PFK production significantly. The ATP-PFK enzyme was purified by desalting (ultrafiltration) chromatography, affinity chromatography and anion exchange chromatography. The purification of ATP-PFK from *L.antarcticum* yielded 2.85 mg of purified PFK enzyme at a purification fold of about 1.3 with specific activity of 5.92 mU/mg and recovery of 7 %. The ATP-PFK activity of *L.antarcticum* was assayed spectrophotometrically by the coupling method monitoring the oxidation of NADH at which will decrease the absorbance at 340 nm for 5 min at 5 $^{o}$C. The purified PFK was homogenous on SDS polyacrylamide gel with an estimated molecular mass of 100 kDa. Gel filtration chromatography showed that the native PFK enzyme is a homodimer and had a molecular weight of approximately 200 kDa. The purified PFK had an optimal temperature at 5 $^{o}$C with optimum pH of 7.0. $Mn^{2+}$, $Co^{2+}$, and $K^{+}$ cation (25 Mm respectively) were found to activate PFK activity, whilst $Cu^{2+}$, $Ca^{2+}$, $Fe^{2+}$, $Zn^{2+}$,

and $Ni^{2+}$ inhibited PFK activity. The substrate specificity of the purified PFK of *L.antarcticum* was specific for F-6-P as phosphoryl acceptor. The nucleotide specificities concluded that *L.antarcticum* PFK as an ATP-dependent enzyme. The purified PFK had an apparent $K_m$ values as determined by kinetic curve for F-6-P and ATP were 41.67 mM and 52.63 mM respectively. The corresponding apparent $V_{max}$ values for F-6-P and ATP were 13.77 mU/mL and 12.12 mU/mL respectively.

# CHAPTER ONE

# INTRODUCTION

## 1.1 General Introduction

Psychrophilic organisms are important in global ecology as a large proportion of our planet is cold. Two-third of sea water covering more than 70% of planet earth is cold deep sea water with temperature around 2 $^{o}$C and more than 90% of freshwater is in polar ice-sheets and mountain glaciers (Kohshima, 2000). Psychrophiles are extremophilic organisms that are capable of growth and reproduce in cold temperatures. Psychrophilic are microorganisms that thrive through low temperatures. These microorganisms have very low optimum growth temperature in the range of 10 $^{o}$C to 20 $^{o}$C (Morita, 1975). They can be contrasted with thermophiles, which thrive at unusually hot temperatures. Despite the fact that a much greater proportion of the earth environment is cold rather than hot, much less is known about psychrophilic, cold-adapted microorganisms compared with thermophiles living at high temperature (Russell, 2000).

To enable them to survive and grow in cold environment, psychrophiles have evolved a complex range of adaptations to all of their cellular components, including their membranes, energy-generating systems, protein synthesis machinery, biodegradative enzymes and the components responsible for nutrient uptake (Russell *et al.,* 1998).

ATP-dependent phosphofructokinase (ATP-PFK, EC.2.7.11) is an intracellular enzyme that catalyzes the irreversible phosphorylation of fructose-6-phosphate (F-6-P) to fructose-1,6-biphosphate (F-1,6-BP) (Hansen and Schonheit, 2000) using ATP as the phosphoryl donor and $Mg^{2+}$ as co- factor. PFK is a key regulatory and definitive enzyme of sugar degradation via the Embden-Meyerhof pathway in all domains of life (Verhees *et al.,* 2001). The Embden-Meyerhof or glycolytic pathway is nearly ubiquitous in all life forms and enzymes of this reaction sequence are highly conserved. PFK is present within the three domains of Bacteria, Eukarya, and Archaea (Ding *et al.,* 1999). The reaction of PFK enzyme is essentially non-reversible step in the glycolytic pathway. The glycolytic and gluconeogenetic pathways of Bacteria and Eukarya are well described, but there is minor knowledge about the carbohydrate metabolism in Archaea.

Apart from its merits as basic science, PFK is also interesting from a biomedical point of view as it is responsible for genetic disorders in some ethnic groups (DiMauro *et al.,* 1997). The industrial application of PFK enzyme have not been fully exploited because of its high cost, as well as lack of sufficient knowledge about its basic characteristics, physiochemical characteristics, regulation mechanism, and optimal conditions.

Yeasts are growth form of eukaryotic microorganisms classified in the kingdom Fungi. Among the fungi PFK that have been purified are species of *Saccharomyces cerevisiae* (Uyeda, 1979), *Yarrowia lipolytica* (Flores *et al.,* 2005), *Kluyveromyces lactis* (Bar *et al.,* 1997), *Pichia pastoris* (Kirchberger *et*

*al.,* 2002), and *Schizosaccharomyces pombe* (Reuter *et al.,* 2000). Since, only a few yeasts PFK have been purified, the search has been done for yeasts that produce PFK enzyme from cold adapted environment.

In this study, the PFK producing yeast *Leucosporidium antarcticum* was isolated from a freshwater sample collected at Davis Station in Antarctic. The PFK enzyme was later purified to homogeneity and its biochemical characteristics was analyzed. Available literatures on cold adapted PFK enzyme are very scarce and therefore need a proper research in order to enhance the enzyme production especially in our own local climate and environments. This entire project is interesting, feasible and has a good potential for growth.

## 1.2 Objectives of Research

The main objectives of this research were to purify the cold adapted PFK enzyme to homogeneity and thus to characterize the enzyme kinetically. Realizing the importance of PFK enzyme, the present investigation was designed with aims to achieve a few objectives:

1. To isolate, screen, identify and classify the potential PFK enzyme producing yeast strain that was isolated from the freshwater sample collected from Davis Station in Antarctic.

2. To study PFK activity from the selected yeast strain (PI12, identified as *L.antarcticum*). The PFK production and cell growth profiles were monitored and determined.

3. To produce PFK from the potential *L.antarcticum* isolates by using fermentable sugar (glucose) as substrate.

4. To enhance the production of PFK from *L.antarcticum* by optimization of cultural conditions and medium composition in a complex medium by using batch culture cultivation approach.

5. To purify the PFK produced by the yeast *L.antarcticum* to homogeneity by chromatography.

6. To characterize the PFK enzyme via kinetic studies and on its significant activity properties.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Psychrophiles

Psychrophilic microorganisms hosts of permanently cold habitats, produce enzymes which are adapted to work at low temperatures. Psychrophiles present in alpine and arctic soils, high-latitude and deep ocean waters, Arctic ice, glaciers, and snowfields. There are generally two groups of psychrophiles; obligate psychrophiles and the facultative psychrophiles. Obligate psychrophiles are those organisms having an optimum growth temperature of 15 $^{o}$C or lower and cannot grow in a climate beyond a maximum temperature of 20 $^{o}$C. These psychrophiles are largely found in icy places such as in Antarctica or at the freezing bottom of the ocean floor (Reddy *et al.,* 2003). Facultative psychrophiles or also referred to as psychrotrophs can grow at 0 $^{o}$C up through approximately 40 $^{o}$C and exist in much larger numbers than obligate psychrophiles. Facultative psychrophiles generally unable to grow much below 0 $^{o}$C though may maintain basic functioning. Facultative psychrophiles have evolved to tolerate cold but are not as physiologically specialized as obligate psychrophiles and are usually not found in the very coldest of environments (David, 2004).

Psychrophilic enzymes display a higher catalytic efficiency over a temperature range of roughly 0-30 $^{o}$C and a high thermosensitivity when compared to their mesophilic counterparts (Gerday *et al.,* 1997). Enzymes from psychrophilic microorganisms operate at temperatures close to 0 $^{o}$C where the activity of the mesophilic and thermophilic counterparts is drastically reduced.

Psychrophiles are characterized by lipid cell membranes chemically resistant to the stiffening caused by extreme cold and often create protein 'antifreezes' to keep their internal space liquid and protect their DNA even in temperatures below water's freezing point (Skidmore *et al.,* 2000). Psychrophiles are of particular interest to astrobiology, the field dedicated to the formulation of theory about the possibility of extraterrestrial life and to geomicrobiology, the study of microbes active in geochemical processes (Richard and Craig, 2004).

## 2.2 Molecular Adaptations of Enzymes from Psychrophilic Organisms

It has generally been assumed that thermophilic is associated with rigid proteins whereas psychrophilic enzymes have a tendency to be more flexible (Aghajari *et al.,* 1998). At the enzymatic level, psychrophilic organisms have to cope with the reduction of chemical reaction rates induced by low temperatures in order to maintain adequate metabolic fluxes. Thermal compensation in cold adapted enzymes is reached through improved turnover number and catalytic efficiency. This optimization of the catalytic parameters can originate from a highly flexible structure which provides enhanced abilities to undergo conformational changes during catalysis (Feller and Gerday, 1997).

Molecular mechanisms of cold adaptation studies examining the molecular mechanisms of cold adaptation in archaea have been performed mostly with M. burtonii. Proteins from the cold-adapted archaea had a higher content of non-charged polar amino acids, particularly glutamine (Gln) and threonine (Thr) with a lower content of hydrophobic amino acids particularly leucine (Leu) (Thomas and Cavicchioli, 2000).

## 2.3 General Properties of Cold-Adapted Enzymes

Psychrophilic organisms from Polar Regions permanently face temperatures ranging from 2 to -2.2 $^{o}$C as in the case of Antarctic sea-water or even lower temperatures for microorganisms growing at the surface of the ice shell. These species have to compensate for the reduction of chemical reaction rates inherent to low temperatures. Indeed, for most biological systems including the single biochemical reaction, any temperature decrease of 10 $^{o}$C results in a 2 to 3 times lower reaction rate (Feller *et al.,* 1997). Nevertheless, the generation times of psychrophilic bacteria near 0 $^{o}$C are of the same order as those of mesophilic microorganisms at 37 $^{o}$C (Feller *et al.,* 1994; Mohr and Krawic, 1980; Morita, 1975). This clearly indicates that mechanisms of temperature compensation are involved.

Cold enzymes produced by psychrophilic microorganisms have three general characteristics in common:

1. A curve of activity as a function of temperature shifted towards low temperatures.
2. A specific activity $k_{cat}$ or physiological efficiency $k_{cat}$ / $K_m$ higher than mesophilic counterparts over a temperature range of 0-30 $^{o}$C.
3. A limited thermal stability illustrated by their fast denaturation at moderate temperature (Feller *et al.,* 1996).

The dominating character of cold-adapted enzymes is probably their enhanced turnover number and catalytic efficiency that compensate for the reaction rate reduction at low temperatures in order to maintain adequate metabolic fluxes (Arpigny *et al.*, 1997; Low *et al.,* 1973). According to the current hypothesis, this optimization of the catalytic parameters can originate from highly flexible structure of these proteins which provides enhanced abilities to undergo conformational changes during catalysis at low temperatures (Hochachka and Somero, 1984; Somero, 1995). The usually observed thermal lability is therefore regarded as a consequence of this folding flexibility. However, one cannot exclude that, besides the structural flexibility imposed by catalysis, the lack of thermal selective pressure also involved in heat lability (Feller *et al.,* 1997).

The relative rigidity and the improved packing density of proteins from thermophiles are frequently proposed as the main structural determinants of their stability (Fontana, 1991; Jaenicke, 1991; Wrba *et al.,* 1990). In addition, Vanhove *et al.,* (1995) have recently observed a clear-cut inverse relation between the flexibility and the stability in three mesophilic ß-lactamases. Unfortunately, such accurate determination of the flexibility is still lacking in psychrophilic enzymes. However, the low stability of psychophilic enzymes has been well demonstrated by the drastic shift of the apparent optimal temperature of activity by the low resistance to protein denaturants and by the susceptibility of secondary structures to unfolding at moderate temperature (Feller *et al*, 1992; Hochachka and Somero, 1984**;** Kobori *et al.,* 1984). A preliminary circular dichroism (CD) studies of psychrophilic and mesophilic $\alpha$-amylases

suggested that the conformation of the cold adapted enzyme was less compact at all temperatures used. In addition, both psychrophilic and mesophilic enzymes seemed to possess the same conformation at their physiological temperature (Feller *et al.,* 1992).

### 2.3.1 Structural Factors Affecting the Stability of Cold-Adapted Enzymes

Molecular modeling of the 3-D structure of the cold adapted enzymes such as bacterial subtilisin from *Bacillus* (Davail *et al.,* 1994), α-amylase from *Alteromonas haloplanctis* (Feller *et al.,* 1994), triose-phosphate isomerase from *Moraxelia* (Rentier-Delrue *et al.,* 1993), β-lactamase from *Psychrobacter immobilis* (Feller *et al.,* 1995) and trypsin from Antarctic fish *Notothenia rossii* reveals that only subtle modifications of their conformation can be related to the structural flexibility. The studies on *M. burtonii* and *H. lacus-profundi* indicate that a general feature of cold adaptation in psychrophiles might be to increase the abundance of unsaturated lipids at low temperature to ensure that membrane fluidity and thereby membrane function is maintained.

These adaptations of protein molecule favor a less compact conformation through a discrete balance between stabilizing and destabilizing factors. Electrostatic noncovalent weak interactions and the hydrophobic effect contribute to the net free energy of stabilization of protein structure whereas destabilizing forces mainly favor the conformational entropy of the unfolded state (Creighton, 1991; Privalov, 1979; Privalov and Gills, 1988).

### 2.3.2 Structural Basis for Cold Adaptation

The molecular mechanisms of adaptation of microorganisms to extreme conditions are of great interest regarding both basic knowledge and development of biotechnologies. Cold adapted microorganisms are potentially useful sources of cold-active enzymes (Russell, 2000). In order to survive in extreme environments, enzymes from psychrophiles must catalyze efficiently at low temperatures. While good progress is being made to elucidate the adaptation mechanism of enzymes from some extremophiles including hyperthermophile, the molecular basis of cold adaptation of psychrophilic enzymes is relatively poorly understood. However, psychrophilic enzymes have generated considerable interest since they can be used to improve the efficiency of industrial processes and for environmental applications. Also, comparison of the structures of psychrophilic enzymes with mesophilic, thermophilic, and hyperthermophilic counterparts may add new insights into the understanding of catalytic mechanism and analysis of thermostability factors (Kim *et al.,* 1999).

## 2.4 Application of Cold-Adapted Microorganisms

### 2.4.1 Biotechnological Potential of Cold-Active Enzymes

The two properties of cold-active enzymes that have the most obvious biotechnological application are their high catalytic activity at low temperatures and low thermostability at elevated temperatures. Ohgiya *et al.,* (1999) have described three groups of enzymes according to their thermolability and catalytic properties. The majority of enzymes belong to either group 1 or 2. Group 1 comprises those enzymes that have similar activity and more heat-

sensitive than the equivalent mesophilic enzymes, whereas group 2 enzymes have higher activity at low temperature and more heat sensitivity. In comparison, enzymes with higher activity at low temperature but similar thermostability (group 3) are rare (Cavicchioli *et al.*, 2002).

The properties of cold-active enzymes provide numerous avenues for industrial application. However, specific properties may be improved through enzyme engineering. This may include enhancing inherent properties, such as increased thermolability and catalytic activity at low temperatures or the modification of pH profiles or other biochemical properties. Generating a thermostable enzyme while retaining high catalytic activity (group 3) would see applicaton of 'cold-active' enzymes in high temperature processes. This latter class has been generated using site-directed mutagenesis and directed evolution. In addition, these methods have been used to increase the activity of thermostable enzymes at low temperatures while fully or partially retaining their thermal stability (Cavicchioli *et al.*, 2002).

Cold-adapted enzymes offer economic benefits through energy savings: they negate the requirement for expensive heating steps, function in cold environments and during the winter season, provide increased reaction yields, accommodate a high level of stereospecificity, minimize undesirable chemical reactions that can occur at higher temperatures and exhibit thermal lability for rapidly and easily inactivating the enzyme when required (Gerday *et al.,* 1997; Russell, 2000). The ability to heat-inactivate cold active enzymes has particular relevance to food industry where it is important to prevent any modification of

the original heat sensitive substrates and product. This is also of benefit in sequential processes (molecular biology) where the actions of an enzyme need to be terminated before the next process is undertaken; with cold adapted enzymes this might be accomplished by heat inactivation rather than chemical extraction. Cold-active enzymes also find application in mixed aqueous-organic or non-aqueous solvents for the purpose of organic synthesis. Their utility derives from their inherent flexibility, which counteracts the stabilizing effects of low water activity in organic solvents (Gerday *et al.,* 1997).

Despite their biotechnological potential and in comparison with the use of thermostable enzymes, few cold-adapted enzymes are in commercial use. Example of commercial applications includes a protease from Novozyme sold as an encapsulated detergent. Researchers in Japan at the Hokkaido National Industrial Research Institute have isolated organisms from cold soil and water that have yielded a cold-active protease from *Pseudomonas* PL-4 and a cold-active lipase from *Typhica ishibariensis* (Joseph *et al.,* 2007). The Norwegian company Biotec ASA has exploited its location near the marine environment of North Sea to isolate cold-adapted enzymes.

The list of enzymes from Biotec include shrimp alkaline phosphatase, cod uracile-DNA glycosylase (a recombinant protein), lysozyme from Arctic scallops, cod pepsins (for the hydrolysation of proteins, production of caviar and descaling of fish) and an enzyme for the efficient skinning of squid (Gomes and Steiner, 2004).The EU Fourth Framework research programme has sponsored a project to examine enzymes from Antarctica, some of which may

have commercial potential. Enzymes arising from this work include α-amylase (used in breadmaking, textiles, brewing and detergents), cellulose (used in textiles and the pulp and paper industries), β-galactosidase (which eliminate lactose from milk), lipase (used in detergents and flavourings), protease (used in detergents and meat tenderizing) and xylanase (breadmaking) (Margesin and Schinner, 1999). Genencor, in USA, is a leader in the development of novel enzymes for industrial applications and claims that its Optisize Amylase and IndiAge Super cellulose are active at low temperature but do not indicate if the enzymes are derived from cold-adapted organisms. The Organisation for Economic Cooperation and Development has published a book comprehensively examining the application of enzymes across a broad range of industrial applications with a view to creating clean industrial processes.

There has been little in the way of patent protection for enzymes from psychrophilic organisms. Most activity has been from Japan with the Japan Advanced Institute of Science having two patents for cold active proteases CP-58 and CP-70 (Kulakova *et al.,* 1999). Another Japanese company, Kao Corporation has filed a number of patents for cold active proteases for use in detergent. This patent describes the protease and the organism that produces them. Another patent from the University of Liege, describes a cold-active β-galactosidase with activity down to 8 $^{o}$C, but the patent does not describe what it is used for (Francois *et al.,* 2001).

### 2.4.2 Protein Production

Genes encoding cold-active enzymes from bacteria have been cloned and expressed in *E. coli* grown at low temperature (Russell, 2000; Truong *et al.,* 2001; Hoyoux *et al.,* 2001). Over expression at temperature below 30 $^{o}$C in *E. coli* result in less inclusion bodies and an increased recovery of soluble protein, however the rate of production and yields may be reduced.

Antarctic bacteria have been screened for characteristics that may enable the development of low-temperature recombinant gene expressions systems for heat labile proteins (Remaut *et al.,* 1999). These characteristics included optimal growth temperature and ranges of growth temperature, survival after cryopreservation, natural resistance to antibiotics and transformation efficiency of several broad host range plasmids. Bacterial strains TA1 and TAD1 exhibited high gene transfer (electroporation and conjugation) efficiencies, and induction levels of up to 250 fold were achieved at 4 $^{o}$C and 15 $^{o}$C (Remaut *et al.,* 1999). A cold adapted amylase was over expressed and post translational processed in the psychrophile *Pseudoalteromonas haloplanktis* TAC125 (Tutino *et al.,* 2001). Expression was nearly two orders of magnitude lower than that obtained in *E. coli* grown at 18 $^{o}$C. However, with further optimization, this is a promising system for expression of heat-labile cold-adapted protein. Cold active PFK enzymes were also used in regulating carbohydrate catabolism and anaerobic energy metabolism of cardiac preservation in the absence of oxygen (Carpenter and Hand, 1986).

### 2.4.3 Other Application of Cold-Adapted Microorganisms

In addition to their enzymes, cold-adapted microorganisms have a range of demonstrated and potential applications. The Archaea are a novel and untouched biotechnological resource. Archaea synthesize a range of cellular products not found in Bacteria and Eucarya. In addition, Archaea often colonize extreme environment and this is reflected in the novel products they produce. Several cold-adapted Archaeal products have potential biotechnological application. These products include enzymes (Hough and Danson, 1999), metabolic cofactors (methanopterin in methanogens), light-harvesting complexes (bacteriorhodopsin from extreme halophiles) and ether-linked lipids. An interesting example of the use of ether-linked lipids is their application in the production of liposomes for vaccine and drug delivery (Patel and Sprott, 1999).

A diversity and abundance of Archaea exist in cold aquatic environment (Cavicchioli *et al.,* 2002). In view of their unusual properties, the cold-adapted Antarctica Archaea *Methanogenium frigidum*, *Methanococcoides burtonii* and *Halorubrum lacussprofundii* provide important sources for biotechnological discovery. Limited work has been carried out occurred in this field (Cavicchioli *et al.,* 2002), however it has already been shown that the psychrotolerant strain *M. burtonii* accumulates potassium aspartate during low-temperature growth and this has been shown to decrease the $K_m$ for the binding of GTP (Thomas *et al.,* 2001). Because of the lack of Archaeal expression systems, cold-active gene products from Archaea have been expressed exclusively as recombinant protein in *E. coli* (Thomas *et al.,* 2001; Schleper *et al.,* 1997; Thomas and Cavicchioli, 2000). Optimal expression for EP-2 from *M. burtonii* was achieved

by growing *E. coli* at 37 $^{o}$C and inducing expression at 14 $^{o}$C for 16 hours. An obstacle encountered in heterologous expression systems is disparity in codon bias, although this has been corrected by co-transformation with plasmids encoding minor tRNAs (Thomas and Cavicchioli, 2000). Archaeal proteins that require modified amino acids, post-translational processing (proteolytic cleavage, methylation) or incorporation of metal centres will require expression systems in Archaea. Expression systems exist for halophilic Archaea and genetic systems have been developed for two genera of methanogenic Archaea (Sowers and Schreier, 1999). The system developed for *Methanosarcina acetivorans* has a broad host among *Methanosarcinsal* species and preliminary studies indicate that the vectors may be transformed into *Methancoccoides methylutens* paving the way for a similar in *M. burtonii*.

The perspectives of biocatalysts have been markedly widened due to investigation into various areas including studies on enzymatic apparatus of extremophilic microorganisms, the most numerous group of which are psychrophiles (>80% of biosphere is the psychrosphere, characterized by an average temperature below 5 $^{o}$C). Though cold-adapted enzymatic proteins synthesized by psychrophiles act according to the same catalytic mechanisms as homologous enzymes produced by other organisms (Feller and Gerday, 1997) they display some specific kinetic and structural features which confer efficient function in energy-deficient environments (Gerday *et al.,* 1997).

Enzymes from Antarctic psychrophilic bacteria are the main objective of studies in the field of low-temperature biocatalysts (it is believed that true

psychrozymes are synthesized by microorganisms which permanently exist at low temperatures). On the other hand, only two enzymes from Antarctic yeast, such as an aspartyl proteinase from a psychrophilic strain of *Candida humicola* (Ray *et al.* 1992) and a xylanase from psychrophilic strain of *Cryptococcus adeliae* (Petrescu *et al.* 2000) have been purified and characterized to date. An extracellular serine proteinase from psychrophilic marine yeast *Leucosporidium antarcticum* is the third reported enzyme of that type (Turkiewicz *et al.,* 2003). It is a particularly interesting enzyme since it is produced by the Antarctic endemic species of yeast (Fell *et al.,* 1969).

## 2.5 Glycolysis

The Embden-Meyerhof or glycolytic pathway is nearly ubiquitous in all life forms and enzymes of the reaction sequence are highly conserved. One of the key and definitive enzymes of the pathway is PFK. PFK is present within the three domains Bacteria, Eukarya, and Archaea (Ding *et al*., 1999). PFK catalyzes the irreversible phosphorylation of F-6-P to F-1,6-BP (Hansen & Schonheit, 2000). Glucose is a key metabolite in organism's metabolism. Glycolysis is the bio-chemical pathway of glucose degradations is known to play an important role in energy metabolism. The process results in the net synthesis of two molecules of adenosine triphosphate (ATP) per consumed molecule of glucose. Glycolysis accomplishes the degradation of glucose to pyruvate. Its main purpose is the generation of energy (ATP). Glycolysis generates some ATP directly and more indirectly by way of subsequent oxidation of pyruvate. The need for ATP is universal, so that the glycolytic

pathway is found in every cell of any organisms. Glycolysis pathway is a central to metabolisms other pathways lead into and out of it.

Its central position in metabolism suggests that it must have an early origin in the development of life. In the absence of oxygen, it is the main way of generating energy in the living cell. Energy is conserved in the pathway by coupling the redox reactions to a cofactor (NAD) and also by coupling the oxidation to phosphorylation and dephosphorylation leading to the net accumulation of ATP. Figure 2.1 shows all the enzymes involved in the catalytic reactions in glycolytic pathway (Ma and Zeng, 2003).

Glycolysis involves 10 enzymatic reactions as summarized below:-

1. The phosphorylation of glucose by hexokinase.
2. The isomerization of glucose-6-phosphate to fructose-6-phosphate by phosphohexose isomerase.
3. The phosphorylation of fructose-6-phosphate by phosphofructokinase.
4. The cleavage of fructose-1,6-bisphosphate by aldolase. This yields two different products, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate.
5. The isomerization of dihydroxyacetone phosphate to another molecule of glyceraldehyde phosphate by triose phosphate isomerase.
6. The dehydrogenation and phosphorylation of glyceraldehyde-3-phosphate to 1,3-bis-phosphoglycerate by glyceraldehyde-3-phosphate dehydrogenase.
7. The transfer of the 1-phosphate group from 1,3-bis-phosphoglycerate to

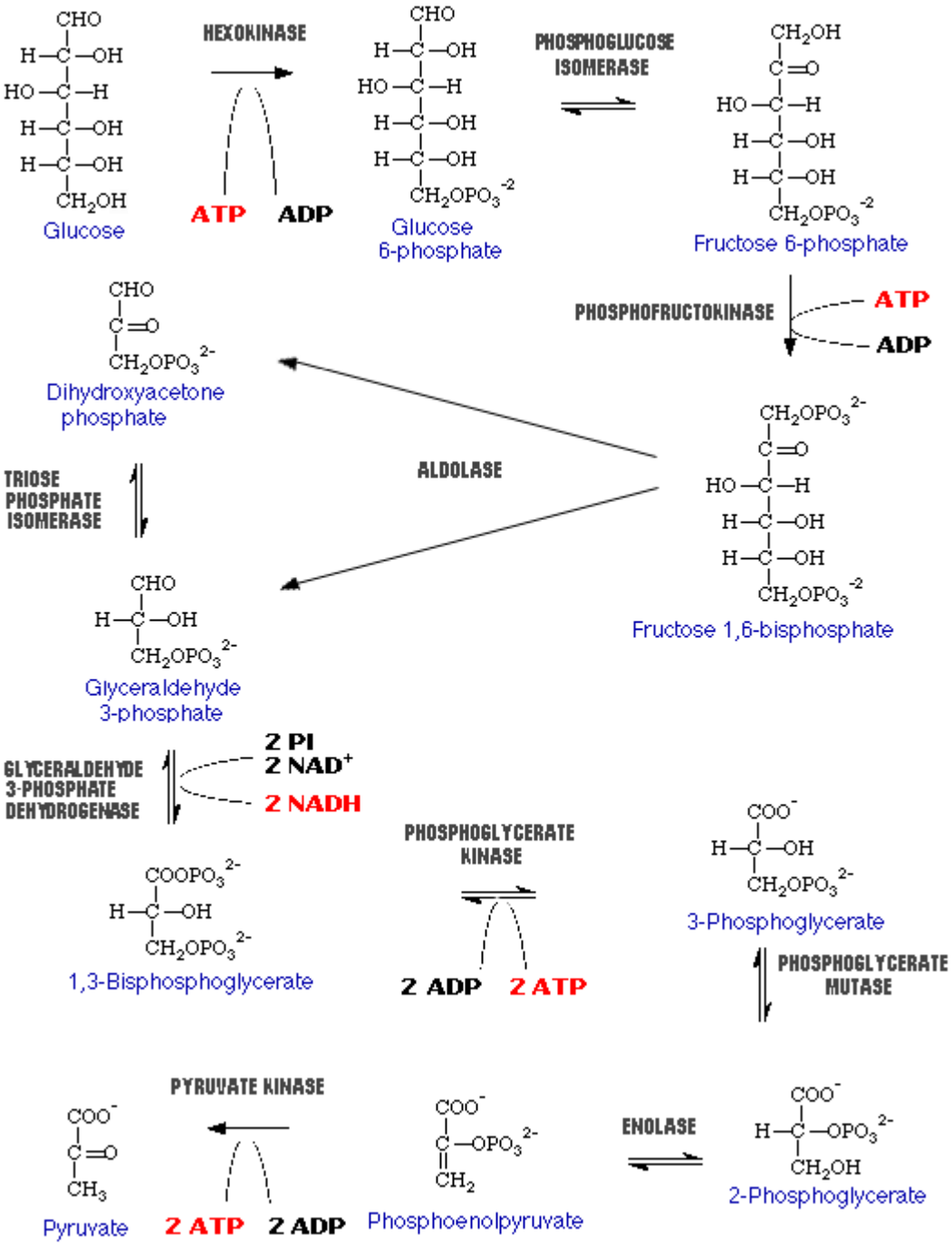


**Figure 2.1: Glycolytic pathway. Also shown are the enzymes involved in the catalytic reactions (adapted from Ma and Zeng, 2003).**

ADP to yield ATP by phosphoglycerate kinase which yields 3-phosphoglycerate.

8. The isomerization of 3-phosphoglycerate to 2-phosphoglycerate by phosphoglycerate mutase.
9. The dehydration of 2-phosphoglycerate to phosphoenolpyruvate by enolase.
10. The transfer of the phosphate group from phosphoenolpyruvate to ADP by pyruvate kinase to yield another ATP.

## 2.6 Phosphofructokinase (PFK)

PFK is the most important regulatory enzyme (EC 2.7.1.11) of glycolysis. Figure 2.2 shows PFK catalyzes the physiologically irreversible phosphorylation of F-6-P to F-1,6-BP (Hansen and Schonheit, 2000). It is an allosteric enzyme made of 4 subunits and controlled by several activators and inhibitors. This leads to a precise control of glucose and the other monosaccharides, galactose and fructose going down the glycolysis pathway. PFK catalyzes the first committed step of glycolysis, since it is not only irreversible but also because the original substrate is forced to proceed down the glycolytic pathway after this step (Knowles, 1980). Before PFK enzyme's reaction occurs, glucose-6-phosphate can potentially travel down the pentose phosphate pathway or be converted to glucose-1-phosphate and polymerized into the storage form glycogen.

In part because of the irreversible nature of this step in glycolysis, PFK is the key regulatory enzyme for glycolysis. When ATP levels are high in the cell,

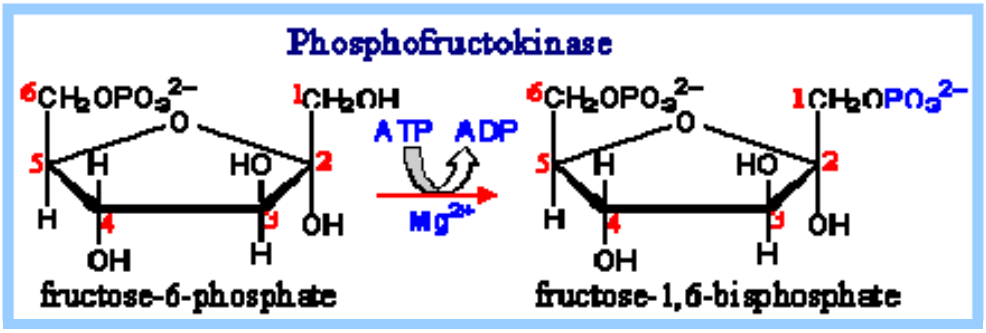


**Figure 2.2: Phosphofructokinase catalyzes the phosphorylation of F-6-P + ATP to F-1,6-BP + ADP**

the cell no longer needs metabolic energy production to occur. In this case, activity of PFK is inhibited by allosteric regulation by ATP itself, closing the valve on the flow of carbohydrates through glycolysis (Saylor, 1998). ATP binds in two places on PFK: in the active site as a substrate and in the regulatory site as a negative modulator. ATP bound in the regulatory site acts as a modulator by lowering the affinity of PFK for its other substrate, F-6-P (Boyer, 1997).

PFK is also inhibited by abundant cellular concentrations of citrate, another marker of a high energy state of a cell. When citrate levels are high, the cell can obtain more than enough energy from the citric acid cycle and does not need glycolysis to shovel more carbons into the citric acid cycle (Boyer, 1997). Figure 2.3 shows the molecular structure of *Bacillus stearothermophilus* phosphofructokinase depicted from Evans and Hudson, 1979.

## 2.7 Subtypes of Phosphofructokinase

In general, ATP is regarded as the universal energy carrier and the most common phosphoryl group donor for kinases. However, several glycol and PFK have been reported to have different phosphoryl group donor specificity. Those enzymes require ADP and PPi as the phosphoryl group donor instead of ATP and are involved in a modified Embden-Meyerhof pathway in this organism.

Three subtypes of PFK with respect to their phosphoryl donor, have been described: an ATP-, an ADP-, and a pyrophosphate-dependent (PPi) form (Ding *et al*., 1999).

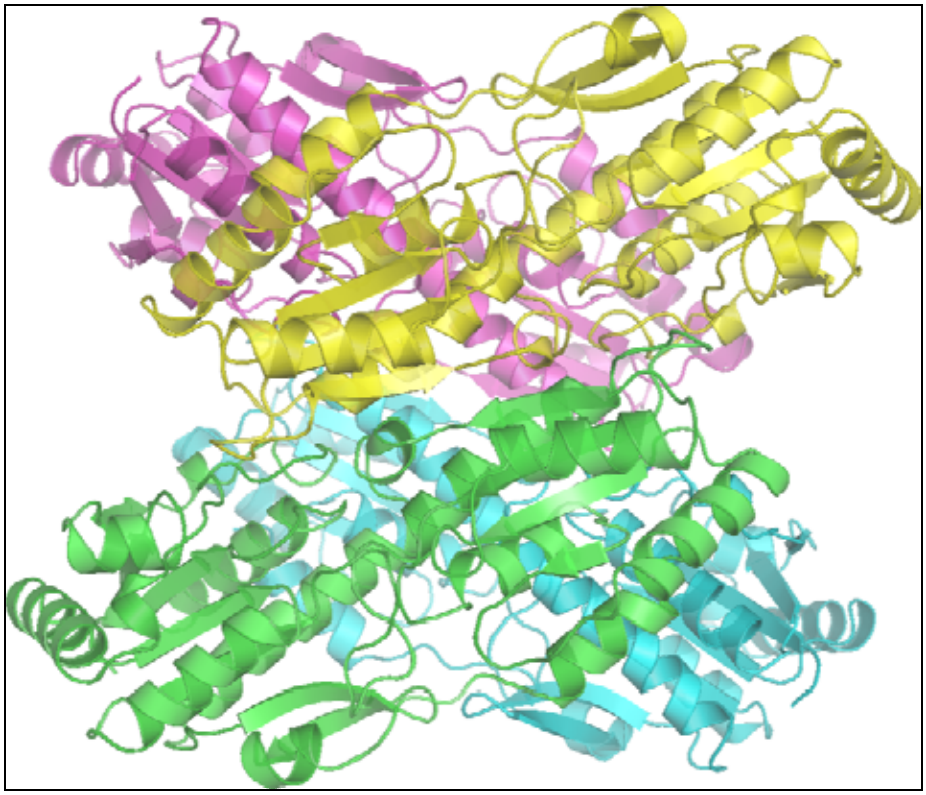

**Figure 2.3: *Bacillus stearothermophilus* phosphofructokinase (Evans and Hudson, 1979).**

### 2.7.1 ATP-Dependent Phosphofructokinase

ATP-dependent phosphofructokinase (ATP-PFK, EC.2.7.11) is a key regulatory enzyme of sugar degradation via the Embden-Meyerhof pathway in bacteria and eukarya (Hansen and Schonheit, 2000). In the majority of organisms, ATP is the phosphoryl donor for the enzyme (Ding *et al.,* 1999). ATP-PFK have been purified and characterized from a variety of bacteria and eukarya (Uyeda, 1979). The ATP-PFK is found in many bacteria (Byrnes *et al.,* 1994), in most highly evolved organisms (Mertens, 1991) and in the crenarchaeon *Desulfurococcus amylolyticus* (Selig *et al*., 1997). The regulation of ATP-PFK activity is usually complex with several positive effectors (such as AMP, cyclic AMP, or ADP) and negative effectors (ATP, citrate, or phosphoenolpyruvate) with the nature of the allosteric modulation being dependent on the source of the enzyme (Ronimus *et al.,* 1999). The ATP-PFK can be broadly classified into bacterial type or the eukaryal type (Ronimus *et al.,* 1999). The bacterial enzymes are generally homotetramers composed of 35 kDa subunits and are activated allosterically by ADP and inhibited by phosphoenolpyruvate (Blangy *et al.,* 1968**;** Evans and Hudson, 1979). The bacterial enzymes are sensitive to a limited number of allosteric effectors (Michels *et al.,* 1997). ATP-PFK from eukaryotes are also generally homotetrameric enzymes with larger number of effectors including citrate and F-2,6-BP (Uyeda, 1979). Although most bacterial and eukaryal ATP-PFK are allosterically controlled, nonallosteric examples have been characterized from *Dictyostelium discoideum* (Martinez-Costa *et al.,* 1994) and in the mesophilic bacterium *Lactobacillus delbrueckii* (bulgaricus) (Le Bras *et al.,* 1991). Thermostable bacterial ATP-PFK or their encoding genes, have been found in