BIOCHEMICAL AND GENETIC STUDIES ON THE PYRUVATE BRANCH POINT ENZYMES OF *RHIZOPUS ORYZAE*

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Approval of the Graduate School of Natural and Applied Sciences.

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I certify that this thesis satisfies all the requirements as a thesis for the degree of Doctor of Philosophy.

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

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ABSTRACT

BIOCHEMICAL AND GENETIC STUDIES ON THE PYRUVATE BRANCH POINT ENZYMES OF *Rhizopus oryzae*

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Rhizopus oryzae is a filamentous fungi which produces lactic acid and ethanol in fermentations. *R. oryzae* has numerous advantages for use industrial production of L-(+)-lactic acid but the yield of lactic acid produced on the basis of carbon consumed is low.

Metabolic flux analysis of *R. oryzae* has shown that most of the pyruvate produced at the end of the glycolysis is channelled to ethanol, acetyl-CoA and oxaloacetate production. This study aimed to answer some questions addressed on the regulation of pyruvate branch point in *R. oryzae* and for this purpose biochemical characterisation of the enzymes acting at this branch point and cloning the genes coding for these enzymes have been done.

Pyruvate decarboxylase was purified and characterised for the first time from *R. oryzae*. The purified enzyme has a Hill coefficient of 1.84 and the K_m of the

enzyme is 8.6 mM for pyruvate at pH 6.5. The enzyme is inhibited at pyruvate concentrations higher than 30 mM. The optimum pH for enzyme activity shows a broad range from 5.7 and 7.2. The monomer molecular weight was estimated as 59±2 kDa by SDS-PAGE analysis.

Pyruvate decarboxylase (*pdc*A and *pdc*B) and lactate dehydrogenase (*ldh*A and *ldh*B) genes of *R. oryzae* have been cloned by PCR-cloning approach and the filamentous fungi Aspergillus niger was transformed with these genes. The *A. niger* transformed with either of the ldh genes of *R. oryzae* showed enhanced production of lactic acid compared to wild type. Citric acid production was also increased in these transformants while no gluconate production was observed

Cloning of hexokinase gene from *R. oryzae* using degenerate primers was studied by the use of GenomeWalker kit (Clontech). The results of this study were evaluated by using some bioinformatics tools depending on the unassembled clone sequences of *R. oryzae* genome.

Keywords: *Rhizopus oryzae*, *Aspergillus niger*, pyruvate decarboxylase, lactate dehydrogenase, hexokinase, pyruvate branch point, L-(+)-lactic acid, glycolysis, purification, PCR-cloning, transformation

ÖZ

Rhizopus oryzae' NIN PİRUVAT AYRIM NOKTASI ENZİMLERİ ÜZERİNDE BİYOKİMYASAL VE GENETİK ÇALIŞMALAR

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Rhizopus oryzae fermentasyon sonunda laktik asit ve etanol üreten bir filamentli küftür. Endüstride laktik asit üretimi için *R. oryzae* kullanımının bir cok avantajı vardır ve günümüzde endüstride L-(+)-laktik asit üretimi için kullanılmaktadır. Kullanılan karbon kaynağına göre elde edilen laktik asit verimi düşüktür.

R. oryzae'da yapılan metabolik akım analizi çalışmalarınında gösterdiği gibi glikoliz sonunda üretilen piruvatın büyük bir kısmı laktik asit üretiminin yanı sıra etanol, asetil koenzimA, ve oksaloasetat üretimine kanalize edilmektedir. Buna dayanarak bu çalışmada *R. oryzae* 'da piruvat ayrım noktasının regülasyonu üzerine kurulan bazı soruların cevaplandırılmasına yönelik ayrım noktasında rol alan enzımlerin biyokimyasal karakterizasyonu ve enzimleri kodlayan genlerin klonlanması çalışılmıştır.

Piruvat dekarboksilaz enziminin *R. oryzae* 'dan saflaştırılması ve karakterizasyonu ilk defa olarak gerçekleştirilmiştir. Saflaştırılmış enzimin Hill katsayısı 1,84 ve K_m değeri, pH 6,5'te 8,6 mM'dır. Enzim, 30 mM'dan fazla piruvat derişimlerinde inhibe olmaktadır. Enzimin optimum pH'sı 5,7 ve 7,2 arasında geniş bir aralıktadır. Monomer moleküler ağırlığı SDS PAGE analizi ile 59±2 kDa olarak bulunmuştur.

R. oryzae'nın piruvat dekarboksilaz (*pdc*A ve *pdc*B) ve laktat dehidrogenaz (*ldh*A ve *ldh*B) genleri PCR-klonlama yöntemi kullanılarak klonlanmış ve filamentli küf *Aspergillus niger* 'a aktarılmıştır. Laktat dehidrogenaz genlerinden herhangi birini taşıyan *Aspergillus niger* transformantlarında orijinal suşa göre daha fazla laktik asit üretimi görülmüştür. Bu transformantlarda sitrik asit üretiminde bir artış görülürken glukonat üretimi görülmemiştir.

R. oryzae'da heksokinaz geninin klonlanması dejenere primerler kullanılarak GenomeWalker Kit ile çalışılmıştır. Bu çalışmanın sonuçları *R. oryzae* genomunun düzenlenmemiş klon dizilimleri kullanılarak değerlendirilmiştir.

Anahtar kelimeler: *Rhizopus oryzae*, *Aspergillus niger*, piruvat dekarboksilaz, laktat dehidrogenaz, hekzokinaz, piruvat ayrım noktası, L-(+)-laktik asit, glikoliz, saflaştırma, PCR-klonlama, transformasyon

To my family...

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ADH	alcohol dehydrogenase
A. nidulans	*Aspergillus nidulans*
A. niger	*Aspergillus niger*
amp	ampicilline
AP1	adapter primer 1
AP2	adapter primer 2
CTAB	cethyltriethyl ammonium bromide
D. occidentalis	*Debaromyces occidentalis*
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
glk	glucokinase gene
GLK	glucokinase
GSP1	gene specific primer 1
GSP2	gene specific primer 2
hxk	hexokinase gene
HXK	hexokinase
K. lactis	*Kluyveromyces lactis*
LDH	lactate dehydrogenase
ldh	*lactate dehydrogenase gene*
leu	leucine
LMP-agarose	low melting point agarose
MMS	minimal medium salt
nic	nicotinamide
o/n	over night
P. angusta	*Pichia angusta*
P. pastoris	*Pichia pastoris*
PCR	polymerase chain reaction
PDC	pyruvate decarboxylase
pdc	*pyruvate decarboxylase gene*
PDH	pyruvate dehydrogenase complex
PMSF	phenyl methyl sulfonyl flouride

PYC	pyruvate carboxylase
pyc	*pyruvate carboxylase gene*
R. oryzae	*Rhizopus oryzae*
S. cerevisiae	*Saccahromyces cerevisiae*
S. ventriculi	*Sarcina ventriculi*
TAE	Tris acetate EDTA buffer
TE	Tris HCl EDTA buffer
TPP	thiamine pyrophosphate (cocarboxylase)
Z. mobilis	*Zymomonas mobilis*
Z. palmae	*Zymobacter palmae*

CHAPTER 1

INTRODUCTION

Understanding the physiology and revealing the regulatory metabolisms are the main objectives of many ongoing biotechnological researches on the industrial organisms. Usually the main aim in these studies is to find a way to increase the yield of an industrial product. In this respect glycolysis is one of the main targets being the central metabolic pathway which lies at the heart of many other pathways leading to various products (Figure 1.1)

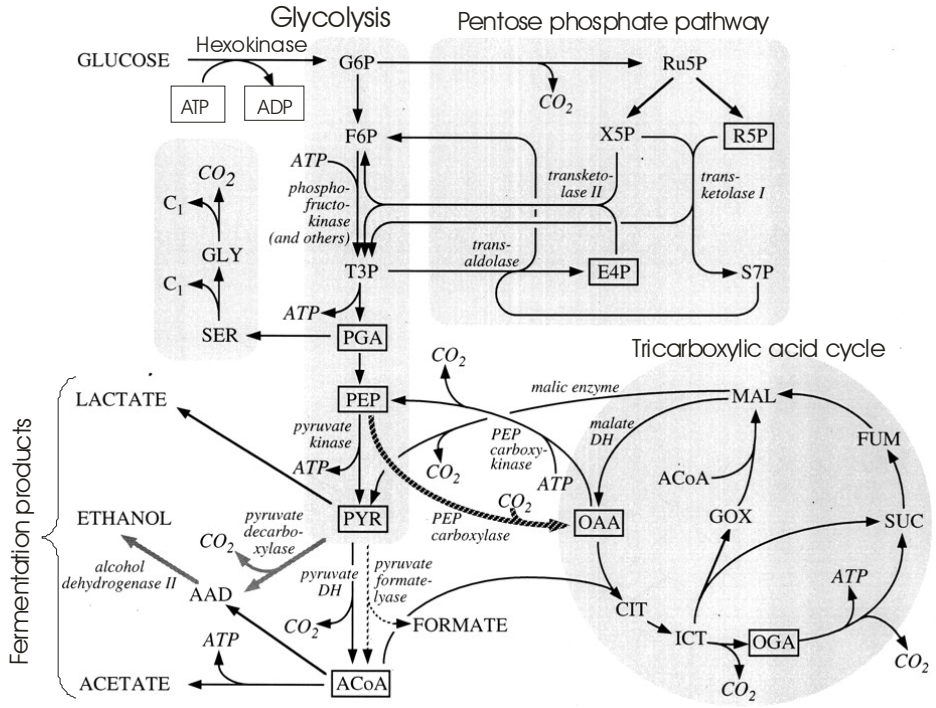

Figure 1.1: The glycolytic pathway and branching pathways. The scheme is showing the central metabolic pathway of *Escherichia coli* and is taken from Sauer *et al.* (1999, Journal of Bacteriology, vol 181, 6679-6688).

Glycolysis is the widely studied pathway in plants, fungi, yeast and other eukaryotes. Most of the studies are focused on increasing the flux rate by overexpressing one or more enzymes of the glycolytic pathway [1-3]. Others study metabolic engineering which use analytical methods to identify the regulation of the glycolysis and to quantify the flux at different physiological conditions [4-7]. Biochemical characterisation of the pathway enzymes and expression analysis of the genes coding for these enzymes are important in this sense to supply the data in order to be able to do metabolic engineering on the glycolytic pathway.

Pyruvate branch point as well as glycolysis is the subject of many researches since it lies at an important point where the pyruvate formed at the end of glycolysis can proceed to enter into respiration and/or to other pathways leading to various products. Pyruvate has many fates (Figure 1.2); it can be converted to oxaloacetate, to acetyl Co A either directly via pyruvate dehydrogenase complex (PDH) or via a reaction sequence involving pyruvate decarboxylase (PDC) -aldehyde dehdrogenase - acetyl Co A synthetase, or it can be directed to lactic acid production in some microorganisms.

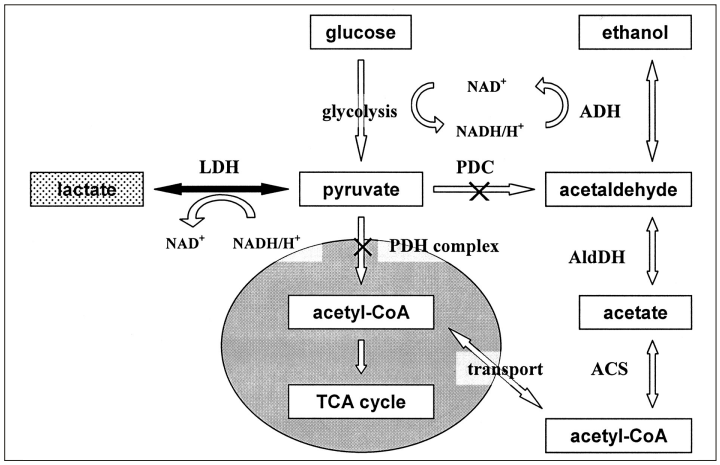

Figure 1.2: Scheme of pyruvate branch point. Enzymes: ACS, acetyl-CoA synthetase; ADH, alcohol dehydrogenase; AldDH, aldehyde dehydrogenase; LDH, lactate dehydrogenase; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase. The scheme shows the modified pathways in *Kluyveromyces lactis* and is taken from Bianchi *et al.*(2001, Applied and Environmental Microbiology, vol 67, 5621-5625).

Channelling pyruvate into the most favourable branch is the main aim in most studies carried out at pyruvate branch point of various organisms. Deletion of one of the genes coding for the branch point enzymes, overexpressing the genes coding for the favourable branch or while deleting one gene, overexpressing the other gene has been subject of a number of studies [8-13].

In the present study, regarding the importance of glycolysis and pyruvate branch point in the metabolism of industrially important microorganism *Rhizopus oryzae* some questions and problems were addressed about pyruvate branch point and glycolysis in this organism. The study also involved another industrially important microorganism *Aspergillus niger* which was used as an expression host for the cloned genes from *R. oryzae*. The proceeding paragraphs will explain the subject organisms, the questions addressed for understanding the pyruvate branch point metabolism of *R. oryzae* and the underlying information found in the literature.

1.1. The organisms: *Rhizopus oryzae* and *Aspergillus niger*

Fungi have been important in biotechnological processes. Processes and products utilising fungi include baking, brewing, and the production of antibiotics, alcohols, enzymes, organic acids, and various pharmaceuticals. *Rhizopus oryzae* and *Aspergillus niger*, both of these organisms subject to this study are filamentous fungi. More descriptive information about both *R. oryzae* and *A. niger* is as follows.

1.1.1. *Rhizopus oryzae*

Rhizopus oryzae is a filamentous microfungi. Its full lineage is given as Eukaryota; Fungi/Metazoa group; Fungi; Zygomycota; Zygomycetes; Mucorales; Mucoraceae; *Rhizopus* (www.ncbi.nlm.nih.gov). It has many synonyms: *R. tritici*, *R. thermosus*, *R. tamarii*, *R. suinus*, *R. peka*, *R. hangchow*, *R. formosaensis*, *R. formasaensis* var. *chylamydosporus*, *R. delemar*, *R. chiuniang*, *R. arrhizus*, *R. liquefaciens*, *R. javanicus* Y. Takeda, *R. pseudochinensis* (www.ncbi.nlm.nih.gov). The group in which *R. oryzae* belongs to, Zygomycetes, is named after the zygospores which are formed during the sexual cycle of the organisms belonging to

this group. Zygospores are formed by a process initiated by fusion of two opposing strains of different mating type [called (+) and (-) [14]. Asexual reproduction is characterised by sporangia in which non-motile spores (sporangiospores) are produced [14]. Another characteristic of this group is that the organisms of this group lack regular septation in their hyphae, in other words they are coenocytic. This is one of the reasons why they are classified as lower fungi in some of the references [15].

Rhizopus oryzae is an obligate aerobe that is often used for industrial production of L-(+)-lactic acid that has an estimated global market of 100,000 tons per year [16]. The produced lactic acid is used mainly in food industry as an acidulant for flavour or as an antimicrobial agent. Its use in the form of nonchlorinated solvent, ethyl lactate and the biodegradable plastic poly-lactic acid gives it potential to increase in the amount of industrial production. Lactic acid is also produced by lactic acid bacteria (mainly *Lactobacillus* species) but *R. oryzae* fermentations are preferred over these fermentations since it produces solely the L-(+)-lactic acid form streospecifically. L-(+)-lactic acid is the preferred form of lactic acid since it could be metabolised in the human body. Another advantage over lactic acid bacteria fermentations is that *R. oryzae* can grow in a chemically defined minimal medium which lowers the costs as well as it makes the downstream processing of the lactic acid easier.

Most of the *Rhizopus* species were isolated as active component in the production of oriental foods like tempe and alcoholic beverages in Indonesia, China and Japan. It was reported to convert glucose to a large amount of L-(+)-lactic acid in 1930s [17, 18]. It is also known that some strains produce fumaric acid as the abundant organic acid species [19-23].

1.1.1.1. Physiology and genetics of *Rhizopus oryzae*

Rhizopus oryzae ferments glucose into lactic acid and ethanol in a typical fermentation. When the lactic acid production of this organism is compared with that of lactic acid bacteria it is far below the level of lactic acid bacteria in that they have at least 95 % yield on the basis of the glucose converted into lactic acid. A typical lactic acid yield which could be obtained in *R. oryzae* fermentations is about 65 % [24].

The regulation of the production of ethanol or lactic acid by this fungus attracts many researchers since it would be a step forward in trying to manipulate the fungal metabolism to produce the favourable product. In trying to understand the regulation of its metabolism lactate dehydrogenase (*ldh*A and *ldh*B) and pyruvate decarboxylase (*pdc*A and *pdc*B) genes of *R. oryzae* were cloned and characterised [16, 25]. Purification and characterisation of the lactate dehydrogenase enzyme from *R. oryzae* were done as well [26, 27].

Chitosan which is a second valuable by-product after *R. oryzae* fermentations were also evaluated [28-30]. Chitosan is the primary cell wall material of this organism (cited in, [31]) thus the 'no more useful' biomass of the organism could be used as a source of production of chitosan which is commercially produced from shellfish chitin.

Apart from the cloning of some genes there were not many studies done on the genetics of *R. oryzae*. However, there are increasing number of studies done on this organism regarding its physiology and biochemistry since it can be used and is used as a producer of lactic acid, fumaric acid, lipase and xylanases [18, 21, 23, 32-34]. Increasing number of studies on *R. oryzae* makes it necessary to be able to transform this organism since it is somehow limiting to only study the biochemistry of this organism. Some of the published studies were based on cloning of *R. oryzae* genes and transforming *Saccharomyces cerevisiae* with these genes e.g, lactate dehydrogenase (*ldh*A) gene [11], lipase gene [35]. In addition to *S. cerevisiae*, the methylotropic yeast *Pichia pastoris*, was used as a host for the high level production of *R. oryzae* lipase as well [36]. The most advantageous side of transforming *S. cerevisiae* with these genes is that genetics of *S. cerevisiae* is very well known, various mutant strains are available and transformation of this organism is very well established. *Pichia pastoris* is an organism which has a well established transformation system and is used as an expression host for intracellular and extracellular proteins from different sources [36]. Regarding the well established systems of these organisms, a reason for not overexpressing the cloned genes in *R. oryzae* become clearer. There was not any transformation system available for this fungus until recently [37]. The transformation system described is based on the transformation of the *R. oryzae pyr*G mutant by microprojectile particle

bombardment of the ungerminated spores. The transformation of the fungus with this system was successful. However, as stated by the author, there are problems consisting of the mitotic instability of the introduced DNA or lack of the ability to target integration into specific loci [37]. These problems are drawbacks of the transformation systems of many Mucorales fungi such as *Mucor circinelloides* [38-40], *Absidia glauca* [41, 42],and *Phycomyces blakesleeanus* [43, 44]. In addition to developments for a transformation system for this fungus the genome sequencing project is carried out by Whitehead Institute in California, USA. The shot-gun sequences, the unassembled sequences, of the clones are available on the web site of National Center for Bioinformatical Information (NCBI) www.ncbi.nlm.nih.gov/traces. The assembly will be soon available on the web which will further accelerate the research done on this organism.

1.1.2. *Aspergillus niger*

Aspergillus niger is very well known citric acid producer. The lineage of this organism can be given as: Eukaryota; Fungi/Metazoa group; Fungi; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiales; Trichocomaceae; mitosporic trichocomaceae; *Aspergillus* (www.ncbi.nlm.nih.gov). *Aspergillus niger* bearing numerous cross walls (septa) is classified as a higher fungi. [15]

Aspergilli are wide spread in the environment, they occur in soil, on plants, in the air, on foods and decaying matter. Ability of *A. niger* to produce organic acids and various carbohydrate degrading enzymes makes it's a favourite industrial strain used for production of many commercial enzymes and organic acids in bulk amounts. Some of the industrial enzymes obtained by *A. niger* can be listed as α-amylase, cellulase, α-galactosidase, β-glucanase, invertase, pectinase, xylanase, lipase, glucose oxidase. Citrate is almost associated with the name of this fungus and gluconic acid is another organic acid species produced in *A. niger* fermentations.

1.1.1.1. Physiology and genetics of *Aspergillus niger*

A typical *A. niger* fermentation medium contains mainly gluconic acid, citric acid, fumaric acid, oxalic acid and malic acid. *A. niger* was reported to be a very efficient oxalic acid producer with a production of 13 gram oxalic acid l^{-1} from 20 gram sugar l^{-1} (cited in. [45]). Oxalic acid and gluconic acid are not the wanted species in a citric acid production process, regarding this a mutant which is both oxalate and gluconate non-producer was shown to produce an increased amount of citric acid [45].

The citric acid accumulation and its regulation in *A. niger* have some clues to understand the basis of the production although there are still more to discover. Some of the factors and phenomena associated with citric acid accumulation are glycerol accumulation of glycerol in the early stages of growth [46], a high glycolytic flux [47], which is characterised by high level of fructose-2,6-bisphosphate [2, 48], a change from pentose phosphate pathway to glycolysis. Glycerol, which functions as an osmoregulator diffuses slowly out of the cells and possibly into the mitochondria inhibiting the nicotine adenine dinucleotide phosphate (NADP+)-specific isocitrate dehydrogenase (Figure 1.3) [46].

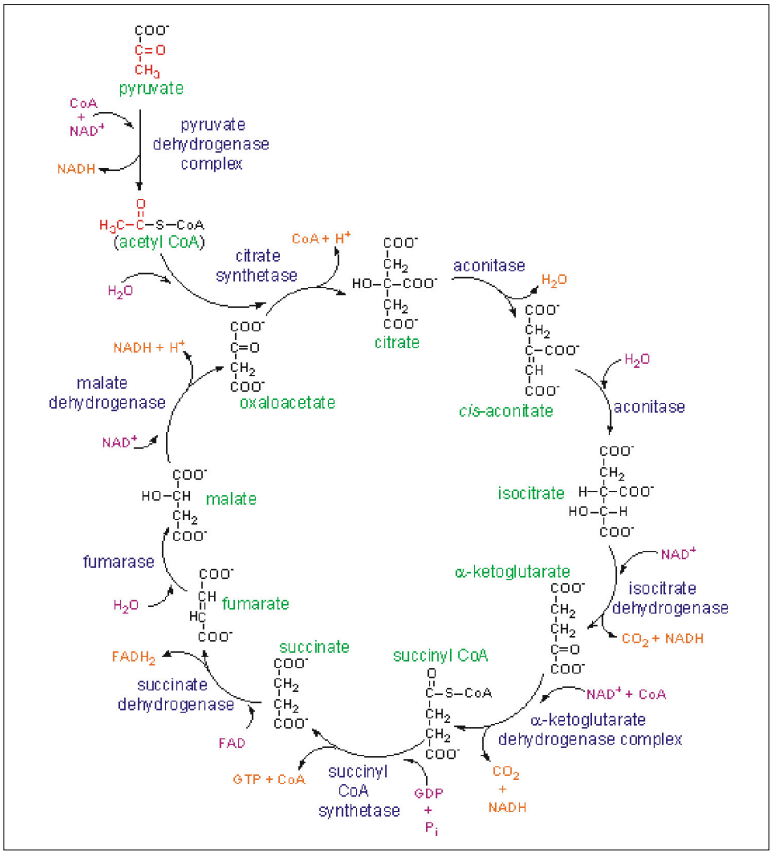

Figure 1.3: Tricarboxylic acid cycle. The scheme shows the basic reactions of the cycle.

Mutants of *A. niger* which were found to produce increased citric acid production were shown to have increased glycolytic capacity associated with an increase in the two glycolytic enzymes, hexokinase and phosphofructokinase [47]. Phosphofructokinase and pyruvate kinase were subjected to an overexpression study and findings showed that there were increase in neither the citric acid accumulation nor in the levels of the other enzymes and intermediary metabolites. However, it was found out that fructose-2,6-bisphosphate levels of the transformants overexpressing phosphofructokinase and pyruvate kinase were almost 2 fold lower compared to wild type strain of *A. niger* representing the high glycolytic flux is strongly regulated by this positive allosteric regulator of phosphofructokinase [2].

Transformation of *Aspergillus niger* is very well established and various different selection systems were proved to be successful. Some of these transformation systems make use of *arg*B (coding for omithine transcarbamylase) [49], *nia*D (nitrate reductase) [50], *amd*S (acetamidase) [51]. As it was reported in these studies there are no problems like mitotic instability of the transformants. The plasmid integration which was reported to be problematic for *R. oryzae* is not a problem for *A. niger* transformations. Protoplast formation is the first step in *A. niger* transformations and the plasmid carrying the genes is introduced with the aid of PEG (polyethylene glycol). A commercial supply, Novozyme 234®, which is a mixture of enzymes containing mainly glucanase is used for degrading the cell wall of *A. niger*. The complete genome of *A. niger* is sequenced and a large number of EST sequences are publicly available (http://aspergillus-genomics.org/asper.fls/asper_blast_dbs.html).

1.2. Pyruvate branch point

Pyruvate branch point where the pyruvate is channelled to a number of pathways (Figure 1.2). The regulation at this important junction determines the yield of many products e.g. lactate, ethanol and by-products like acetate and succinate in industrial fermentations carried out with various industrial microorganisms. Although the pyruvate branch point of many other organisms have been subject to various physiological and genetical studies. For example in plants the regulation at this branch point is important for the long term survival under low oxygen conditions [52-58]. Pyruvate branch point of the other organisms like *Zymobacter palmae* and *Zymomonas mobilis* deserves attention since these organisms can be used for production of ethanol from different sources [59-63]. Among all the other industrial organisms the pyruvate branch point of *Saccharomyces cerevisiae* has taken a considerable attention from the scientists.

Why pyruvate branch point especially in *Saccharomyces cerevisiae* take the attention of many researchers? *Saccharomyces cerevisiae*; "Baker's yeast" is an important industrial microorganism as well as being a model organism for scientific

researches. During the production of baker's yeast, the costs of the carbohydrate feedstock are a major factor in overall economy of the process. Thus a high biomass yield on the sugar feedstock (usually molasses) is a major optimisation criterion. This implies that fermentative sugar metabolism, which leads to a lower biomass yield than respiratory metabolism should be avoided during the production phase. On the other hand, commercial baker's yeast should have a high fermentative capacity in the dough application. During the industrial production of bakers' yeast, the strong inclination of *S. cerevisiae* to perform alcoholic fermentation is largely overcome by careful manipulation of the rate of sugar supply and by controlling other environmental conditions.

Pyruvate branch point reactions in *R. oryzae* also attracts attention since this organism produces both ethanol and lactic acid during fermentations. As indicated earlier in section 1.1.1.1. the drawback of *R. oryzae* fermentations is the low yield of lactic acid on the basis of carbon consumed and in trying to increase the yield one of the possibility could be disrupting one of these branches which is not favourable. For example in trying to increase the lactic acid yield the pyruvate consumed through the ethanol branch could be directed to the lactic acid formation and this may be achieved by disrupting the pyruvate decarboxylase gene(s). A recent study done on strain improvement of *R. oryzae* has shown that the key areas where most of the carbon is lost from lactic acid formation are reactions from pyruvate to acetyl CoA, oxaloacetate and to ethanol [4]. The change in these fluxes can be achieved by genetically modifying the corresponding enzymes such as pyruvate decarboxylase, alcohol dehydrogenase and pyruvate dehydrogenase complex. Not having a well defined transformation system for *R. oryzae* is limiting such type of modifications but there are some studies done on other organisms which are parallel with this theory. To illustrate modification of the pyruvate branch of a wild type and a mutant strain of *S. cerevisiae*, the latter lacking the *pdc*1 were transformed with the *ldh* gene encoding a bovine lactate dehydrogenase and differences in ethanol and lactate production of these organisms were observed [8]. The mutant strain lacking *pdc*1 had a lactate yield of 0.20 g g^{-1} while that of wild type was 0.155 g g^{-1} on the basis of glucose consumed. Ethanol yield of wild type was higher than

(0.35 g g^{-1}) that of mutant strain (0.20 g g^{-1}) suggesting the pyruvate which could be used in pyruvate decarboxylase reaction could have been directed to lactate branch [8].

1.2.1. Enzymes of pyruvate branch point

The enzymes which lie at the pyruvate branch point (Figure 1.2) have been subject to many studies in search for understanding the regulation at this junction and to modify the branch so that one of the branches is favoured in production of the desired product. The enzymes that use pyruvate as substrate are pyruvate decarboxylase, lactate dehydrogenase, pyruvate dehydrogenase complex and pyruvate carboxylase. Having the above mentioned enzyme activities *R. oryzae* accumulates both lactic acid and ethanol in fermentations. The most relevant branch enzymes for lactate and ethanol production, pyruvate decarboxylase and lactate dehydrogenase have been the first two candidates in search for the understanding of the regulation of the partitioning between the lactate and ethanol branch in *R. oryzae*.

1.2.1.1. Pyruvate decarboxylase

Pyruvate decarboxylase (PDC) catalyses the conversion of pyruvate to acetaldehyde in the presence of Mg^{2+} and thiamine pyrophosphate (TPP). The *S. cerevisiae* pyruvate decarboxylases, the most extensively studied pyruvate decarboxylase enzymes are basis of many similar studies on other organisms.

Active PDC in *S. cerevisiae* is a tetramer of a molecular mass of about 250 kDa composed of four identical subunits. This tetramer is composed of two dimers that bind to each other rather loosely, while the dimer itself is very stable. The structure depends on the pH; at pH 6.2 the enzyme exists solely as a tetramer and at pH 8.4 only as a dimer. Between these two pH values the enzyme goes through equilibrium with different proportions of dimers and tetramers. When it is taken into consideration that cytosolic pH is 6.8-6.9 this behaviour of the enzyme will be important in the regulation of the enzyme and as a result the regulation of pyruvate branch point. It has been speculated that when pyruvate is accumulated in the

cytosol, the pH decreases, this will favor the active tetrameric form but the physiological importance of this behaviour is not known. However, pyruvate has a direct effect on the enzyme in terms of substrate activation. Thus the structural and regulatory properties of PDC may well contribute to the preference for pyruvate being metabolised via PDC during alcoholic fermentation [64].

Thiamine diphosphate (cocarboxylase) and Mg^{2+} are the two cofactors of PDC. They are bound very tightly, but not covalently to the enzyme at physiological conditions. Each PDC tetramer contains four molecules of Mg^{2+} and TPP.

Yeast PDC is activated by its substrate and it has been shown that a cysteine residue is likely to be involved in this process. It has been proposed that this cysteine residue form an intramolecular signalling pathway leading to conformational changes at the active site, which in turn, cause activation of the enzyme as soon as the substrate interacts with cysteine [64].

Yeast PDC appears to be a phosphoprotein whose one serine residue being phosphorylated per protein dimer. The relevance for this phosphorylation is unclear, because after incubation with alkaline phosphatase, which removes phosphate group from the residue, PDC activity did not show any alterations [64].

In *S. cerevisiae* a total number of six pyruvate decarboxylase genes (*pdc*) have been described; *pdc*1-6. *pdc*1, *pdc*5 and *pdc*6 encode structural genes for pyruvate decarboxylase while *pdc*2, *pdc*3 and *pdc*4 are thought to code for proteins that are involved in the control of expression of these structural genes. Structural genes for pyruvate decarboxylase have been isolated from a couple of other fungal organisms, as well as from the bacteria *Zymomonas mobilis* [62] *Zymobacter palmeae* [65] and from maize [66]. All pyruvate decarboxylase genes are homologous. Most similar to the *S. cerevisiae* protein is that of the distantly related yeast *Kluyveromyces lactis* with 86 % identity and 94 % similarity [64]. All other known pyruvate decarboxylases are in the range of 30-40 % identical to the yeast enzyme [64]. Two pyruvate decarboxylase genes (*pdc*A and *pdc*B) were cloned from *R. oryzae* and they are similar to each other with 85 % nucleotide sequence identity and 91 % similar on the basis of amino acid identity. Comparison of these genes to other organisms resulted in 29 % similarity to *Zymomonas mobilis pdc* and approximately 40 % to *Aspergillus nidulans* and *Aspergillus oryzae* and all of the

yeast *pdc*. The *pdc*A and *pdc*B from *R. oryzae* were determined to require glucose induction for the initiation of transcription and oxygen availability has influence in regulating the level of *pdc* expression [16].

There are a number of studies done on pyruvate decarboxylase gene deletion mutants and these studies concluded that pyruvate decarboxylase is necessary for growth on glucose since in *S. cerevisiae* strains in which *pdc*1 and *pdc*5 or all three pyruvate decarboxylase genes have been disrupted showed a reduced growth rate in complex (yeast extract-peptone) media [10].

Effects of pyruvate decarboxylase overproduction on flux distribution at the pyruvate branch point in *S. cerevisiae* was also investigated [12]. The results indicate that a limited respiratory capacity was not responsible for the onset of aerobic fermentation in the *pdc*-overproducing strain but *pdc* overprodution affected flux distribution at the pyruvate branch point by influencing competition for pyruvate between PDC and mitochondrial pyruvate dehydrogenase complex (PDH) [12].

1.2.1.2. Lactate dehydrogenase

Lactate dehydrogenase (LDH; EC 1.1.1.27) catalyses the formation of lactate from pyruvate in a reaction which consumes NADH. The reverse reaction is possible as well when consumption of lactate is the case.

The only lactate dehydrogenase genes (*ldh*A and *ldh*B) cloned from a fungus are from *R. oryzae*. Two lactate dehydrogenase genes (*ldh*) were cloned and expression of them under different conditions was studied and as the results indicate the expressions of these two genes are under different circumstances. Evidences were presented so that ldhA is expressed on fermentable sugars and plays a role in lactic acid synthesis. The second gene isolated from *R. oryzae* was *ldh*B which was the species not having the transcripts in the glucose grown cultures. However, *ldh*B transcripts were found in the cultures grown in the presence of glycerol, ethanol, and lactate suggesting that this gene is functional and apparently glucose repressed [25].

In the purification studies of lactate dehydrogenase from *R. oryzae* several authors have described [26, 27] the presence of two lactate dehydrogenase enzymes in *R. oryzae*. An NAD^+-dependent lactate dehydrogenase (EC 1.1.1.27) that converts

pyruvate to lactate but exhibits negligible activity for the reverse reaction is produced during early growth and synthesis of lactic acid. It is suggested that after the glucose is depleted, a second lactate dehydrogenase activity is involved in the NAD^+-independent oxidation of L-(+)-lactic acid to pyruvic acid [26, 27]. In this respect findings of Skory [25] for expression of *ldh*A and *ldh*B support the previous studies [26, 27].

The native NAD^+-dependent lactate dehydrogenase (LDH) from *R. oryzae* is 131 kDa and is a tetramer composed of identical subunits of 36 kDa [27]. The enzyme followed the normal Michaelis-Menten kinetics with a normal hyperbolic saturation kinetics [26]. The K_m for pyruvate was reported as $6.4x\ 10^{-4}$ M [27] and $5.5x10^{-4}$ M [26]. The optimum pH is close to neutral being in the slightly alkaline was reported as 7.2 [26] and as 7.5 [27]. The isoelectric point (pI) of the enzyme is 5.2 [27]. The activity of the enzyme is completely inhibited by Hg^{2+}, Pb^{2+}, Fe^{2+}, Zn^{2+} and Cd^{2+}. [27]. The decrease in the lactic acid production rate of *R. oryzae* by increased levels of Zn^{2+} [20] could be attributed to the inhibition of lactate dehydrogenase activity by this divalent cation.

The effect of NADH on activity of *R. oryzae* LDH was presented as a sigmoidal dependence up to $3x\ 10^{-5}$ M of NADH but above this concentration Lineweaver-Burk plot is linear. The K_m for NADH of this enzyme was reported as $2.9x10^{-4}$ [26] and $1.48x10^{-4}$ [27]. The sigmoidal dependence of the enzyme to varying concentrations of NADH and the slopes of the Hill Plot (n_H) having a value of 1.86 at concentrations up to $3x\ 10^{-5}$ M and 1.04 above this concentration suggests that there are two binding sites for NADH having different affinities towards their substrate [26]. These data were interpreted by the author as that one these two sites has an high affinity for NADH showing a positive cooperativity until saturation but when it is saturated the cooperativity of the binding is not shown [26].

As presented in the above paragraphs the activity of the lactate dehydrogenase enzyme thus the production of lactic acid can be regulated by the changes in pH, NADH and also pyruvate.

A recent study on *R. oryzae* demonstrated that the lactic acid production of this organism can be increased by overexpression of *ldh*A. *R. oryzae* overexpressing

*ldh*A has shown a 7 % increased rate of lactic acid production compared to the wild type. Moreover, there was a decrease in the by-products ethanol and fumaric acid in this transformant [67].

1.2.1.3. Pyruvate dehydrogenase complex

Pyruvate dehydrogenase multienzyme complex occupies a central metabolic position connecting the glycolytic pathway with the main energetic pathway the citric acid cycle. It catalyses the formation of acetyl coenzyme A from pyruvate. Pyruvate dehydrogenase complexes are composed of three constituent enzyme components: pyruvate dehydrogenase (E1), lipoate acteyltransferase (E2), and lipoamide dehydrogenase (E3). The complex has a molecular mass of 7000 kDa to 8500 kDa and consists of multiple copies of E1 and E3 attached around a core of E2 subunits. In mammals and yeast another component called protein X is present. The basic structure of the protein X is similar to E2, it appears to be responsible for the attachment of the E3 subunit to the complex [68].

By the concerted action of three enzymes in the complex the formation of acetyl coenzyme A from pyruvate is catalysed. The functions of the subunits can be listed as E1 (α andβ), pyruvate dehydrogenase (EC 1.2.4.1), E2, lipoate acetyltransferase (EC 2.3.1.12), E3, dihydrolipoamide dehydrogenase (EC 1.6.4.3). In the first step, pyruvate is covalently linked to thiamine pyrophosphate (TPP), the catalytic cofactor of the pyruvate dehydorgenase enzyme yielding 2-α-hydroxy-ethyl-TPP. The hydroxyl group of this active aldehyde is oxidised, and the acetyl group is transferred via lipoamide, the prosthetic group of E2 subunit, to coenzyme A, yielding acetyl coenzyme A. In the final step the oxidised lipoamide is regenerated by the E3 subunit using NAD^+ and FAD as cofactors [64].

The PDH complex isolated from *S. cerevisiae* was shown to have a Km of 0.65 mM for pyruvate at pH 8.1 and a K_m of 0.13 mM at pH 6.5 [69] which indicates that the activity of the enzyme can be regulated by the variations in pH. The optimum pH of the enzyme was shown to be between 7.7 and 8.1. The PDH complexes isolated from *S. cerevisiae* and *Neurospora crassa* has no cooperativity towards pyruvate [70, 71]. Phosphorylation of the E1 subunit of the PDH complex

was shown to inhibit substrate conversion in *S. cerevisiae* [72, 73]. Phosphorylation by kinases and dephosphorylation by phosphatases and the regulation of these enzymes are some of the factors responsible for the activity of the PDH complexes. Direct feedback inhibition of the complex by the products of the overall reaction, acetyl coenzyme A and NADH is another factor affecting the regulation of PDH complex [68].

By introducing gene disruptions in all four structural genes (E1α, E1β, E2 and X) of the pyruvate dehydrogenase complex of *S. cerevisiae*, strains lacking pyruvate dehydrogenase activity were obtained. All of the pyruvate dehydrogenase negative strains were viable in complex and defined media containing either glucose or non-fermentable carbon sources ([74] and reviewed in. [75]). This is not very surprising since in this organism a pyruvate dehydrogenase by-pass which supplies the mitochondria with acetyl coenzyme A is described (reviewed in [64, 75]).

1.2.1.4. Pyruvate carboxylase

Pyruvate carboxylase (6.4.1.1) is a member of the family of biotin-dependent carboxylases and is highly conserved and is found in a great variety of organisms including fungi, bacteria and plants as well as higher organisms. It catalyses the ATP-dependent carboxylation of pyruvate to form oxaloacetate which may be utilised in gluconeogenesis, amino acid, and fat metabolism. The reaction is important in replenishing the TCA cycle intermediates drained off for biosynthesis (reviewed in [75]).

The enzyme consists of four identical subunits arranged in a tetrahedron-like structure. Each ~130 kDa subunit contains a covalently attached biotin prosthetic group which serves as a carrier of CO_2 between the two reaction subsides. The overall reaction catalysed by pyruvate carboxylase (PYC) involves two partial reactions that occur spatially separate subsides within the active site, with the covalently bound biotin acting as a mobile carboxyl group carrier. In the first partial reaction, biotin is carboxylated using ATP and HCO_3^- as substrates whilst in the second partial reaction, the carboxyl group from carboxybiotin is transferred to pyruvate [76, 77].

The availability of biotin is one of the main regulators of PYC activity. Biotinylation of the apo-PYC is important since this is the only way to activate the inactive form. In yeast the long term regulation of the enzyme occurs via biotin availability and the ability of the organism to biotinylate the apo-PYC. Short term regulation of the enzyme occurs by acetyl coenzyme A (activation) and L-aspartate (inhibition) [78, 79].

The disruption of pyruvate carboxylase in *S. cerevisiae* resulted in strains which are unable to grow on a defined medium containing glucose as the sole carbon source. Growth of this pyruvate carboxylase negative mutant was possible when aspartate instead of ammonium was added [80]. This is in consistence with the role of pyruvate carboxylase in the glyoxylate cycle and that it can not be by-passed under the conditions (glucose repressed) tested. However, isolation of a suppressor mutant of a pyruvate carboxylase negative strain, in which the glucose repression of the glyoxylate cycle enzymes is lessened, shows that the inability to by-pass the PYC reaction is because of the regulatory factors rather than physiological ones [75].

1.3. Glycolysis

Glycolysis is the central pathway which is used to metabolise all the sugars aerobically and anaerobically. The pathway is described as a series of reactions starting by phosphorylation of the sugar and ends with the formation of pyruvate (Figure 1.4).

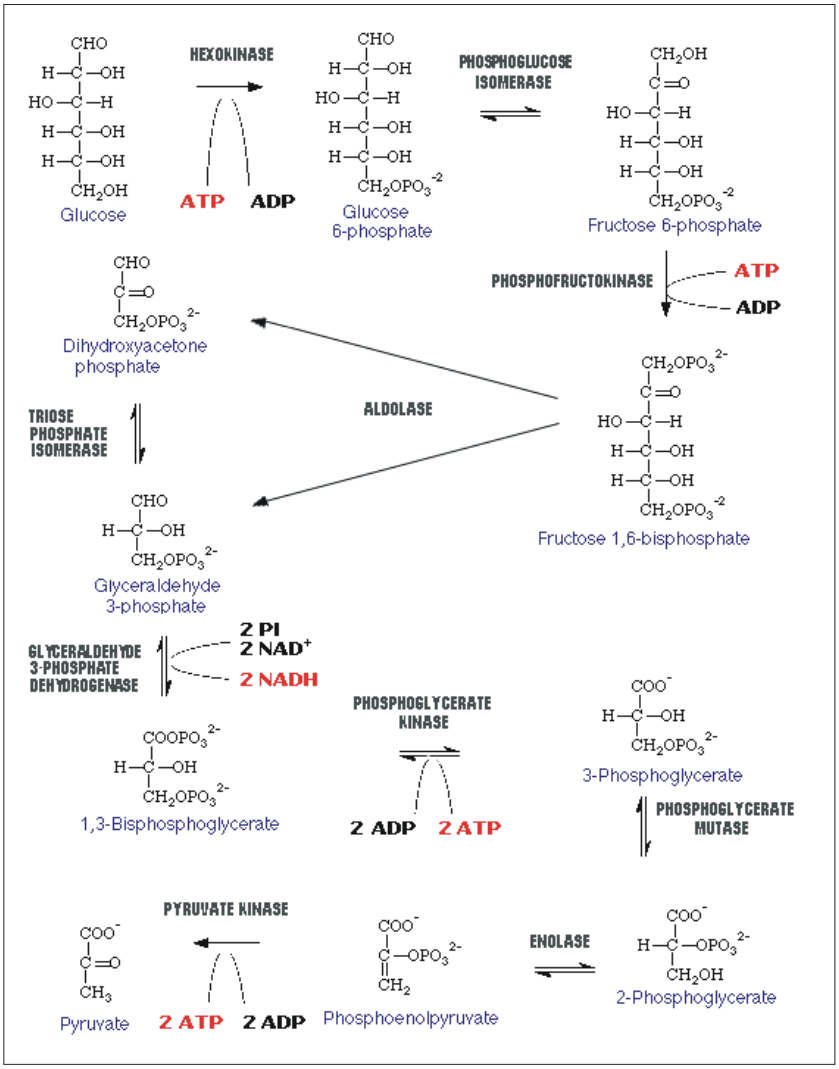

Figure 1.4: The glycolytic pathway

It is commonly found almost exclusively in all of the organisms and since it lies at the heart of complex metabolism extensive studies are being done to better understand the action and regulation of this pathway. From biotechnological point of view understanding regulation of glycolysis is essential in trying to partially control the metabolism and to increase the yield of the processes. Regarding this, glycolysis and its controls have been studied in many organisms.

The first approach of many studies done on glycolysis were focused on finding the so called 'key points' in glycolysis. These key points were claimed to be hexokinase, phosphofructokinase and pyruvate kinase reactions. They were thought

to be the regulating points since these were the irreversible steps of the glycolysis. In trying to determine if these reactions are the limiting reactions overexpression studies of these enzymes were performed. However, over expression of these enzymes were not very successful in that there were strong controls on them by other other factors. For example in *Saccahromyces cerevisiae* strain having an hexokinase-overproduction of even 50 fold did not result in an increased glycolytic flux since there was a control over these mutants which could make use of increased trehalose-6-phosphate levels to inhibit the hexokinase activity. Similarly overexpression of phosphofructokinase and pyruvate kinase in Aspergillus niger were not successful showing that increasing the specific activity of these 'key enzymes' was not enough to increase the glycolytic flux. Strong regulation of phophofructokinase reaction by a nonglycolytic intermediate fructose-2,6-bisphosphate which is a positive allosteric regulator was found to be responsible for the down regulation of phosphofructokinase expression in overexpression strains [2].

The overexpression studies on glycolytic enzymes being not successful led the researchers to overexpressing several of the glycolytic genes simultaneously [1, 3]. These studies are based on the idea that increasing the flux at some point does not necessarily mean that the other steps of the glycolysis could afford these changes. These studies were successful to a limited extend and an increase in in glycolytic flux under enhanced ATP demand [3]. These studies implicated that for a substantial enhancement of the glycolytic flux all enzymes of glycolysis need to be simultaneously overexpressed and that this strategy might fail because of an excessive protein burden (references). An extensive study of the factors influencing the glycolysis is necessary for successful metabolic engineering of the glycoltic pathway of *S. cerevisiae* as well as other organisms. To illustrate the regulation of the glycolysis at the genetical level should be also taken into account since Gcr1p (glycolysis regulation-1) was shown as an important transcription factor in the regulation of the glycolytic genes [81-83].

1.3.1. The first step of glycolysis

Hexokinase catalyses the first and irreversible step of glycolysis (Figure 1.4). Although there are a high number of studies [84] have been done and being done on this enzyme the role of it in the glycolysis and its control have not been clear till now. There are a few metabolic 'roles' attributed to this enzyme one of which is sugar sensing in lower and higher eukaryotes including plants [85-87]. Since hexokinase 2 of *S. cerevisiae* were shown to be able to autophosphorylated it was claimed that it could play role in the intracellular signalling [88, 89].

Hexokinases and glucokinases from several fungi have been purified and characterised including those from *S. cerevisiae* [90, 91], *Aspergillus niger* [92, 93], and Hansenula polymorpha [94]. *S. cerevisiae* have three type of isoenzymes; hexokinase 1, hexokinase 2 [91] and glucokinase [90] as well as *Aspergillus niger* is known to posses a hexokinase [93] and a glucokinase [92]. These isoenzymes are differentiated on the basis of their sugar specificity. For example *A. niger* glucokinase has a K_m of 0.063 mM for glucose while it has a K_m of 120 mM for fructose [92] while that of the hexokinase of the same organism has a K_m of 0.35 mM and 0.2 mM for glucose and fructose respectively [93].

1.4. Aim of the study

Rhizopus oryzae, an important industrial microorganism, which can be used for production of L-(+)-lactic acid has the disadvantage of low lactic acid yield. Our theories were all based on this problem and the ultimate goal of this study was to understand lactic acid metabolism of this fungus in trying to find a way to increase the lactic acid production capacity.

This study is composed of three main parts: 1) Purification and characterisation of pyruvate decarboxylase from *R. oryzae* 2) PCR-cloning of pyruvate decarboxylase (*pdc*A and *pdc*B) and lactate dehydrogenase (*ldh*A and *ldh*B) genes of *Rhizopus oryzae* and expression of these genes in *Aspergillus niger* 3) Genomic library construction from *R. oryzae* and cloning of hexokinase (*hxk*) and glucokinase (*glk*) genes of *R. oryzae*

The first and second parts focus on the pyruvate branch point enzymes and genes of the lactic acid-producing filamentous fungus *R. oryzae*. Both biochemical and genetical approach were used to understand the regulation of pyruvate branch point in *R. oryzae*. The pyruvate decarboxylase and lactate dehydrogenase genes of *R. oryzae* were cloned by PCR-cloning approach. *Aspergillus niger* was used both as an expression host for these genes and to evaluate the effect of introducing these genes into this organism. The last part is composed of genomic library construction and cloning of hexokinase and glucokinase genes from *R. oryzae*. Some tools of bioinformatics were used to evaluate the data obtained at this part. The third part is especially important to be able to work on genetics of *R. oryzae* and in turn is essential in strain improvement of this fungus.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. The strains

Rhizopus oryzae ATCC 9363 was purchased from American Type Culture Collection. *Aspergillus niger* NW 219 strain, was supplied by Wageningen University Fungal Genomics Group. *Escherichia coli* DH5α were used as competent cells for propagation of the constructed plasmids.

2.1.2. The plasmids

pGEM T-easy vector system (Promega) was used for the propagation of the gene fragments to be sequenced. The plasmid pIM 4902 was used as expression vector in *Aspergillus niger* NW 219 transformations. The plasmid maps are given in Appendix A.

2.1.3. The primers

The primers used for generating the *ldh* and *pdc* gene fragments were synthesised by Isogen. The primers used for sequencing experiments were supplied by Westburg Genomics.

2.1.4. The growth media

For growth of *E. coli* DH5α the LB medium (Appendix B) was used. For *R. oryzae* and *A. niger* the growth media are given in Appendix C and D.

2.1.5. The chemicals

The chemicals used in all experiments were all of analytical grade and commercially available from Sigma, Aldrich, Merck, Oxoid and Fluka.

2.2. Methods

2.2.1. Purification and characterisation of pyruvate decarboxylase from *Rhizopus oryzae*

2.2.1.1. Growth of *Rhizopus oryzae*

Rhizopus oryzae ATCC 9363 was allowed to sporulate on solid medium containing 15 mM $(NH_4)_2SO_4$, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 0.2 µM $ZnSO_4$, 28 mM glucose, 2 % (w/v) agar at 30 ˚C for 4 days. Spores were inoculated into 1 litre flasks containing 500 ml complex medium which is composed of 111 mM glucose, 86 mM NaCl, 0.5 %(w/v) yeast extract and 1 % (w/v) peptone. Media inoculated with spores to a concentration of 10^6 spores/ml were incubated in a shaker-incubator 35 ˚C and 175 rpm for 16 hours. The mycelium was harvested by filtration through cheese cloth and washed with extraction buffer (0.1 M potassium phosphate buffer containing, 1 mM EDTA, 1 mM PMSF and 1.4 mM β-mercaptoethanol pH 7.0). The mycelium was used directly for purification of pyruvate decarboxylase.

2.2.1.2. Extraction of proteins from *Rhizopus oryzae*

Four grams of wet weight mycelium was suspended in 45 ml of 0.1 M potassium phosphate buffer pH 7.0 contaning, 1.4 mM β-mercaptoethanol, 1 mM EDTA, 1 mM PMSF. After stirring to make the suspension homogenous, the suspension was sonicated at 15 Watt for 3 minutes as 1 second pulse and 3 seconds

of operation. The preparation was incubated on ice during sonication. The sonicated material was filtered through cheese cloth and centrifuged at 4 ˚C for 30 minutes at 20000 g in a Sorvall RC5C type centrifuge. The pellet was discarded and the supernatant (~40 ml) called as crude extract was used for further steps. All further purification steps were carried out at 4 ˚C.

2.2.1.3. Ammonium sulphate precipitation and dialysis

The first step in purification was ammonium sulphate fractionation. Solid ammonium sulphate was added slowly to the crude extract initially at 35 % saturation with continuous stirring. Crude extract was allowed to stand for 1 additional hour after salt addition and centrifuged at 12 000 g for 20 minutes in Sorvall RC5C type centrifuge. The pellet was discarded and supernatant was brought to 55 % ammonium sulphate saturation and stirred for 1 hour. The solution was centrifuged at 12 000 g for 20 minutes, the supernatant was discarded and pellet was dissolved in minimum amount of 0.01 M imidazole HCl pH 6.3 buffer containing 1.4 mM β-mercaptoethanol, 1 mM EDTA, 1 mM PMSF, 1 mM $MgCl_2$ and 0.1 mM TPP. The protein solution was dialysed against 2 x 2 liters of the same buffer for 24 hours at 4 °C; with one change of buffer. The dialysate was centrifuged at 10000 g for 10 minutes to remove undissolved and denatured proteins, the pellet was discarded and supernatant was used in next step.

2.2.1.4. Gel filtration and anion-exchange column chromatography

After ammonium sulphate precipitation the proteins were separated by gel filtration (Sephadex G150) column (2.2 x 35 cm) equilibrated with 0.01 M imidazole HCl pH 6.3 containing 1.4 mM β-mercaptoethanol, 1 mM EDTA, 1 mM PMSF, 1 mM $MgCl_2$ and 0.1 mM TPP. The flow rate of column was 16 ml per hour; fractions of 3 ml were collected and assayed for PDC activity. The fractions having PDC activity were pooled.

The pooled active fractions of 21 ml were loaded onto DEAE-cellulose column (1.5 x 25 cm) which was equilibrated with 0.01 M imidazole HCl pH 6.3

containing 1.4 mM β-mercaptoethanol, 1 mM EDTA, 1 mM PMSF, 1 mM $MgCl_2$ and 0.1 mM TPP. After washing the column with the same buffer at a rate of 12 ml per hour, a linear gradient of 0 – 0.5 M KCl in a total volume of 400 ml was applied. PDC activity was eluted at 0.35 M KCl. Protein concentrations and specific activities of the active fractions were determined. The fractions with a specific activity higher than 90 U/mg were pooled and kept at –20 ˚C in 20 % glycerol. This enzyme solution was used for partial characterisation of the enzyme.

2.2.1.5. Enzyme assay

Pyruvate decarboxylase enzyme activity was assayed spectrophotometrically by a coupled assay system at 340 nm, which determines NADH consumption. A scheme of the PDC enzyme assay is given in Figure 2.1. The reaction mixture contained 25 mM sodium pyruvate, 5 mM $MgCl_2$, 0.2 mM TPP, 11 U/ml alcohol dehydrogenase and 0.15 mM NADH in 40 mM imidazole HCl buffer pH 6.5 in a total volume of 1.2 ml. The reaction was started by addition of appropriately diluted enzyme solution (~0.01 mg/ml). One unit of enzyme is defined as the amount that converts 1 µmol pyruvate to acetaldehyde in 1 minute at 35 ˚C. The amount of protein in cell extracts was determined by dye-binding method [95].

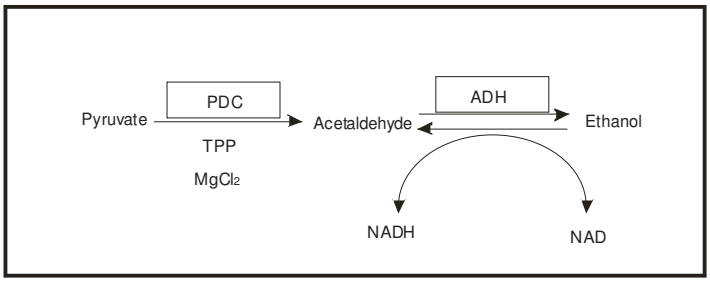

Figure 2.1: A scheme of PDC activity determination. The abbreviations used in the scheme stand for: PDC; pyruvate decarboxylase, ADH; alcohol dehydrogenase, TPP; thiamine pyrophosphate.

2.2.1.6. Characterisation of pyruvate decarboxylase

2.2.1.6.1. Electrophoretic analyses

Native PAGE and SDS-PAGE of purified enzyme was done essentially according to the procedure of Laemmli [96]. SDS PAGE was performed using 4 % stacking and 12 % separating gel. The native gels were prepared with 4 % stacking and a concave pore gradient polyacrylamide gel slabs (5-22.5 %). After electrophoresis, the native gels were stained using two different methods: silver staining [97] and activity staining [98]. The complete protocols are given in Appendix E and F respectively.

2.2.1.6.2. Kinetic analysis

To determine the pH dependency of PDC activity three different buffers were used imidazole-HCl (between pH 6.3-7.7), HEPES (between 6.8-8.0) and MES (between pH 5.5-6.5) and all the other parameters were kept as indicated above. The effect of pyruvate concentration on the enzyme activity was tested using 18 different concentrations between 0.5 and 150 mM. The temperature and pH were kept constant at 35 ˚C and 6.3 respectively in these measurements. The K_m and the cooperativity of the enzyme were calculated from the graphs that were drawn on the basis of the Michaelis–Menten equation and Hill Plot.

2.2.2. PCR cloning of lactate dehydrogenase A (*ldh*A), lactate dehydrogenase B (*ldh*B), pyruvate decarboxylase A (*pdc*A) and pyruvate decarboxylase B (*pdc*B)of *Rhizopus oryz*ae and transformation of *Aspergillus niger* with these genes

2.2.2.1. PCR cloning of *ldh*A, *ldh*B, *pdc*A and *pdc*B from *Rhizopus oryzae*

2.2.2.1.1. Genomic DNA isolation from *Rhizopus oryzae*

The genomic DNA from *R. oryzae* was isolated using a method which is originally for bacterial DNA isolation [99] and which is modified for use in *R. oryzae*

in our lab. The compositions of the solutions are given in Appendix G. To obtain the mycelia for isolation of DNA, *R. oryzae* was grown in a 500 ml liquid rich medium containing 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl, 1 % (w/v) peptone and 2 % (w/v) glucose. The spores were inoculated in a concentration of 10^6 spores/ml and were grown at 35 ºC and 175 rpm for 16 hours. The mycelia were filtered with suction and the pellet was dried between paper towels and was wrapped up in a piece of aluminum foil and was immediately frozen in liquid nitrogen. The mycelia were ground using micro-dismembranator (Braun). The ground mycelia were dispensed into 2-ml eppendorf tubes (100-150 mg per tube). The ground mycelia were suspended in extraction buffer composed of 810 µl TE buffer, 90 µl 10 % SDS (final concentration 1 %) and 9 µl proteinase K (final concentration 0.2 µg/ml) and after mixing thoroughly the suspension was incubated for 1 hour at 37 ºC. At the end of incubation period 162 µl 5 M NaCl was added and mixed thoroughly. Subsequently 270 µl CTAB/NaCl solution was added and the suspension was further incubated for 20 minutes at 65ºC. After addition of 700 µl of chloroform/isoamyl alcohol (24:1) the suspension was spinned for 20 minutes in a microcentrifuge (13 000 rpm: 15 000 g) at room temperature. The aqueous phase was removed to a clean microcentrifuge tube, leaving the interface behind. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and extraction was carried out thoroughly, and the solution was spinned down in a microcentrifuge for 10 minutes. This step was repeated until no more interface is seen between two phases. The extraction is carried out once more with an equal volume of chloroform/isoamyl alcohol to remove any traces of phenol remaining in the aqueous phase. The solution was spinned down in a microcentrifuge for 10 minutes at room temperature and the aqueous phase was taken into a new tube. A 0.6 % volume of isopropanol was added to precipitate nucleic acids. The tube was shaken back and forth until a stringy white DNA precipitate becomes clearly visible. This stringy DNA was either taken out by hooking it out to a fresh tube containing 70 % ethanol or the precipitate was pelleted by spinning briefly at room temperature. The DNA was washed with 70 % ethanol to remove residual CTAB and respinned for 5 minutes at room temperature to repellet it. The supernatant was removed carefully and the pellet was air-dried. The pellet was dissolved in 100 µl TE (10 mM:1 mM) buffer pH 8.0. After the pellet is

incubated overnight to be dissolved completely the solution was treated with RNAase by adding to a final concentration of 20 µg/ml. The quality and concentration of the isolated DNA was checked by running 1 µl and 2 µl (0.1-0.2 µg/µl) of it on a 0.8 % agarose gel along with 0.025 µg, 0.125 µg and 0.250 µg of uncut λ DNA.

2.2.2.1.2. Design of primers for PCR cloning

The primers used for cloning of the lactate dehydrogenase and pyruvate decarboxylase genes of *R. oryzae* were designed based on the sequences available in the NCBI database. Since the gene fragments were cloned into the expression vector, pIM 4902, the restriction sites, *Nsi*I (ATGCA^T) and *Xba*I (T^CTAGA) were introduced respectively at the 5' and 3' end of the gene fragments of *ldh*A, *ldh*B and *pdc*A (Table 3.1). For cloning *pdc*B gene fragment the *Nsi*I and *Hin*DIII (A^AGCTT) sites were introduced at 5'and 3' end of the fragment (Table 2.1), respectively.

Table 2.1: Primers used for PCR cloning of the *R. oryzae ldh*A, *ldh*B, *pdc*A and *pdc*B genes. The restriction enzyme sites that are introduced are underlined in the table.

The gene		The sequence of the primer 5'→3'	Base region for primer	Size of the product
*R. oryzae ldh*A	start	TTCTCATGCATTTACACTCAAAGG	27-50 bp	1100 bp
	end	TAACGTCTAGAATAATATTGTAATTAAAAGTATC	1094-1116 bp	
*R. oryzae ldh*B	start	ATTCCATGCATCTACATTCAAAGG	23-46 bp	1115 bp
	end	ACTCATCTAGATCATAAAATTAGCTACACC	1109-1127 bp	
*R. oryzae pdc*A	start	AAAAACATGCATTCTATTAAAATT	377-400 bp	2064 bp
	end	ATAGGTCTAGAATTGTAACAAATACCCAC	2413-2430 bp	
*R. oryzae pdc*B	start	TAAACATGCATTCTATCCAAATCG	377-401 bp	2015 bp
	end	CAAGCTTGAAGGATTAGGCGCTGCAAAAGC	2362-2384 bp	

The sites introduced were further used to cut out the gene fragment and clone the gene into the expression vector in the right orientation. The *Nsi*I site already contains the ATG start codon and the primer was designed so that *Nsi*I site is placed

at the start codon of the gene(s). The *Xba*I site was introduced at the 3' end of the gene downstream of the stop codon. A few extra bases, which are not matching with the sequence, were added at the 5' end of the end primers. The primers used for PCR cloning of *ldh*A, *ldh*B, *pdc*A and *pdc*B from *R. oryzae* and the regions of the sequence where these primers were based on are given in Table 2.1.

2.2.2.1.3. Isolation of the gene fragments after PCR

The gene fragments were obtained by PCR using *Pfu* Turbo polymerase (Promega), which has 3'→5' proof reading activity. The annealing temperatures and $MgCl_2$ concentrations used for PCR programs were found by optimisation of the PCR at different temperatures (55 °C-65 °C) and containing $MgCl_2$ concentrations(1.5 mM-4 mM). In order to minimise mismatches created by PCR, a proof reading polymerase (*Pfu*) was used. PCR amplification was performed in 30 cycles consisting of 95 ºC for 1 minute, annealing temperature (60 ˚C for *ldh*A and *pdc*B, 62 ˚C for *ldh*B and 63 ˚C for *pdc*A) for 1 minute, and 72 ºC for 1 minute + 7 seconds for *ldh*A, *ldh*B and 2 minutes + 7 seconds for *pdc*A and *pdc*B. The primer concentrations were 10 µM for each set of primers, the concentration of the DNA used as template was 0.1 µg/50 µl reaction mixture. The *Pfu* polymerase used as 1.25 u/50 µl reaction.

The PCR's were carried out in a gradient making PCR thermocycler (Westburg Genomics). After PCR the reaction mixture (25 µl) was run on a 1% agarose gel containing 0.5 µg/ml ethidium bromide along with *Eco*RI/*Hin*DIII-digested λ DNA and 1 kb molecular weight marker (Invitrogen) as molecular weight markers. After verifying that the product obtained is of the right size the band was cut with a sharp scalpel and transferred into an Eppendorf tube. The PCR product was extracted from the gel using the Qiagen gel extraction kit (QIAquick) according to manufacturer's instructions. The method used in this kit is to isolate the DNA from the gel by dissolving the agarose at 50 ˚C and then digesting the agarose with an agarase-containing solution. Once the agarose is digested the DNA is recovered by use of columns that binds DNA onto a silica gel membrane assembly. In the last step DNA is obtained by simply eluting the DNA from the column by 10 mM Tris

HCl pH 8.0 or distilled water. The concentration of the DNA in the solution was estimated by running 3 µl (0.1-0.3 µg) of it on 1 % agarose gel with an appropriate molecular weight marker.

2.2.1.4. Cloning the gene fragments in pGEM T-easy vector and expression in *Escherichia coli*

The *Pfu* polymerase, which was used to generate the gene does not add an adenine base unlike *Taq* polymerase.The fragments obtained by PCR were run on a 1 % agarose gel and were then cut out by use of a sharp scalpel and were extracted from gel slices by use of Qiagen gel extraction kit (QIAquick). In order to clone these blunt ended fragments into PGEM T-easy vector, which uses a T/A cloning strategy, A-tailing procedure was carried out. 0.3-0.7 µg of the fragments was incubated in the presence of 5 units of *Taq* DNA polymerase (Invitrogen), 0.2 mM dATP in the reaction buffer with $MgCl_2$ (supplied with the polymerase), at 70 ˚C for 30 minutes (*Taq* polymerase adds an adenine base at the end of the fragments). Later 0.1-0.2 µg of these fragments were taken and ligated into 50 ng of pGEM T-easy vector in the presence of 5 units of T4 DNA ligase in a reaction volume of 10 µl. The ligation was carried out at room temperature for 3 hours or at 4 ˚C overnight (o/n). Five µl of this mixture containing the plasmid with or without insert were used in transformation of *Escherichia coli* (*E. coli*) as described in Appendix H. Although several colonies were grown on selective media not all of them contained the plasmid with insert. The colonies containing the plasmid with insert appear as white or pale blue colonies which were picked and inoculated for minipreps (Appendix I). The plasmids isolated from these colonies were tested for presence of an insert by digesting 0.1-0.5 µg DNA from each with *Not*I enzyme and run on a 0.8 % agarose gel.

2.2.1.5. Sequencing of the gene fragments

The clones containing the inserts with the expected size were sequenced by GATC-reaction mix (Amersham Pharmacia Biotech) using SP6 and T7 primers. For *pdc*A and *pdc*B another set of primers were used to obtain the whole sequence of the insert. These IRD-labelled (http://www.biolegio.com) primers were based on the 498-514 bp (in reverse direction) and 1113-1130 bp (in forward direction) regions of of *pdc*A and 757-773 bp (reverse) and 2098-2115 bp (forward) regions of *pdc*B (Table 2.2).

Table 2.2: The primers used for sequencing of *pdc*A and *pdc*B

The primers	Label used	Sequence of the primers 5'→3'	Annealing temperature-5˚C
PdcA_reverse	700	AAGTTTATAGGTTCTCG	41
PdcA_forward	700	CAAAAGGCAGTACAAGCC	49
PdcB_reverse	700	AGCTGCTGGTGCTATGC	49
PdcB_forward	700	TGCCAACTCTGAAAACCG	49

The sequencing reactions (PCR) were done in 31 cycles consisting of 95 ºC for 30 seconds, 51 ºC for 30 seconds and 70 ºC for 1 minute.

Depending on the melting temperature of the primers, step 3 was changed and a temperature that is 5 degrees lower than the melting temperature of the primer. Temperature 51 °C was used at step 3 when the sequencing reactions were carried out with SP6 or T7 primers. The duration of step 4 also depends on the size of the fragment and was used as 1 minute for 1000 bp.

2.2.2.2. Transformation of *Aspergillus niger* with *ldh*A, *ldh*B, *pdc*A and *pdc*B of *Rhizopus oryzae*

The *A. niger* strain (NW 219) that is used in this study was derived from N402, a low conidiophore mutant from N400 (CBS 120.49). This strain contains three auxotrophic markers: leucine, nicotinamide and uridine. The media used were based on minimal medium (Appendix D) and were supplemented with 1.5 mM

leucine, 8 µM nicotinamide and 5 mM uridine. *Aspergillus niger*, inoculated at 10^6 spores ml^{-1}, was grown in shake flasks (250 rpm) at 30 ˚C using a starting pH of 6.0.

The transformation strategy is based on co-transformation. The pgw635 plasmid containing the *pyr*A gene that is complementing the uridine auxotrophy, was used as the selection gene DNA. The plasmid pIM 4902 containing one of the genes (*ldh*A, *ldh*B, *pdc*A or *pdc*B of *R. oryzae*) under the control of pyruvate kinase (*pki*A) promoter of *Aspergillus niger* was used as cotransforming DNA. Four different transformation experiments were carried out by using each of these four different genes. The *pki*A promoter is a strong promoter which is induced by glucose. Terminator sequences were of native terminator sequences from *R. oryzae* and were introduced with the PCR-cloned *ldh*A, *ldh*B, *pdc*A and *pdc*B of *R. oryzae*.

2.2.2.2.1. Preparation of expression vector for transformation of *A. niger*

The *ldh*A, *ldh*B, *pdc*A and *pdc*B of *R. oryzae* were digested with the restriction enzymes corresponding to the sites introduced to the 3' and 5' end of the genes. The enzymes used for digestion were *Xba*I, *Nsi*I and *Hin*DIII, and they were used at a concentration of 1U/µl to digest 0.3-0.4 µg of plasmid DNA containing either of the four genes in a reaction volume of 10 µl. The digestion mixture was incubated for four hours at 37 °C. The expression vector was also cut with the corresponding enzymes. The digested fragments were run on 1 % agarose gel and the gene fragments and the vector extracted from the gel by using the Qiagen gel extraction kit (QIAquick).

The gene fragment(s) were then ligated to the expression vector using the T4 DNA ligase. The ligation mixtures included 0.1-0.2 µg of these fragments, 50 ng of pGEM T-easy vector in the presence of 5 units of T4 DNA ligase in a reaction volume of 10 µl. The ligations were carried out at 16 ˚C overnight (o/n). The ligated plasmids were then used to transform *E. coli* DH5α (Appendix H). The transformed *E. coli* clones were used to propagate the plasmid which is then isolated by Wizard® Plus SV Minipreps DNA Purification System (Promega). The system uses the method of alkaline lysis of the cells and then purifying DNA from this solution

through a column. The concentration of the plasmid DNA solution is then checked on agarose gel by running a portion of the plasmid along with *Eco*RI-digested λ DNA as size marker. The concentration of the DNA varied between 35-50 ng/µl for the plasmid minipreps.

2.2.2.2.2. Protoplast formation from *A. niger* NW 219

Two hundred fifty ml of transformation medium (Appendix D) in 1 L flask was inoculated with $1 * 10^6$ spores of *A. niger* NW219 per ml and was grown for 16 - 18 hours at 30 °C and 250 rpm a New Brunswick shaker. The mycelium was harvested on nylon gauze using a Büchner funnel under mild suction, and was washed once with SMC (1.33 M Sorbitol, 50 mM $CaCl_2$, 20 mM MES buffer pH 5.8).

One hundred mg of Novozyme 234® in 10 ml SMC were filter sterilized by passing it through a 0.2 µm membrane filter. One gram (wet weight) of mycelium resuspended in this Novozyme solution by gently pippetting up and down a 10 ml glass pipette.

The suspension was incubated with gently shaking (150 rpm) at 30°C. After 1 h the mycelium was carefully resuspended and a sample was taken to monitor protoplast formation. In case of insufficient amount of protoplasts and the mycelium tips were not degraded incubation was continued for further and was checked every 30 min. When sufficient protoplasts were present (more than $1 * 10^8$) the protoplasts were resuspended carefully in STC and the mycelial debris was removed by filtration over a sterile glass wool plug. The filtrate was collected in a 10 ml glass tube. The protoplasts were centrifuged for 10 minutes at 2000 rpm at 4°C, the pellet was carefully resuspended in 5 ml STC (1.33 M sorbitol in 50 mM $CaCl_2$ and 10 mM Tris/HCl pH 7.5), and were pelleted as above. This wash step was repeated twice and the concentration of the protoplasts was determined before the last centrifugation step, using a heamocytometer (Appendix J). Depending on this count the protoplasts were resuspended in STC at a density of $1 * 10^8$ per ml. The protoplasts were dispersed into 1 ml aliquots and either immediately used for transformation studies or frozen in liquid nitrogen and stored at -70 ºC until use.

2.2.2.2.3. Transformation of *Aspergillus niger*

Two hundred µl of the protoplasts suspension were transferred into a universal tube (screw capped, without a neck and 15 ml of volume), and 1 µg of selection gene DNA, 20-25 µg of co-transforming DNA (dissolved in a 10 - 20 µl TE) were added. A positive (1 µg of selection gene DNA in 20 µl TE (10 mM Tris HCl, 1 mM EDTA, pH 8.0)) and negative control (20 µl of TE) were included and treated as above. Subsequently 50 µl of PEG buffer were added to each transformation tube and mixed gently by shaking followed by incubation at room temperature for 20 minutes. Two ml of PEG buffer (25 % PEG-6000 in TC (50 mM $CaCl_2$ and 10 mM Tris/HCl (pH 7.5)) were added to each tube and mixed gently and incubated at room temperature for another 5 minutes. Finally 4 ml of STC were added and mixed gently by inversions. Selective MMS-top agar (Appendix D), melted and brought to 48 ºC, was added to the transformation mixture so that the tube was almost completely filled. The tube was capped and mixed by inverting the tube several times and was poured onto two 15 cm selective MMS-plates (Appendix D). For the negative control, only half of the mixture was poured on one 15 cm selective MMS-plate. All of the plates are incubated at 30 ºC and the colonies were picked up after 4-5 days.

2.2.2.2.4. Screening for *A. niger* transformants

Transformants were screened for uridine prototrophy. Transformants obtained from uridine-free transformation plates were purified via single spore colonies by plating them on complete medium (Appendix D).

2.2.2.2.4.1. PCR screening of transformants

The transformants were grown in minimal medium (Appendix D) supplemented with 1.5 mM leucine, 8 µM nicotinamide. For growth of wild type 5 mM uridine was included in the medium additionally. The media were inoculated with 10^6 spores/ml and incubated at 30 ºC 250 rpm for 16-18 hours. The mycelium

was then filtered through nylon gauze, dried between paper towels and was frozen in liquid nitrogen. The DNA of the transformants and the wild type was isolated according to the protocol given in Appendix K. Each transformant was amplified using the corresponding primer set which were used for PCR cloning of the genes: *ldh*A, *ldh*B, *pdc*A and *pdc*B from *Rhizopus oryzae*. The PCR mixture was prepared as given in section 2.2.2.1.2. but for convenience instead of *Pfu* polymerase *Taq* polymerase was used at a concentration of 0.2 U/µl. A positive control with *R. oryzae* DNA and a negative control with *A. niger* NW219 DNA were set as well. Once the reactions were over the reaction mixtures were run on 1 % agarose gel to check if there were products and to check the sizes of the products.

2.2.2.2.4.2. Measurement of enzyme activities of transformants

The transformants which were determined to contain the genes (*ldh*A, *ldh*B, *pdc*A or *pdc*B) were then analysed for enzyme activity. The transformants were inoculated at a concentration of 10^6 spores/ml into 250 ml flasks containing 70 ml minimal medium (Appendix D) supplemented with 0.1 % casamino acids, 0.1 % yeast extract, Vishniac solution (1 ml in 1 L), 1.5 mM leucine, 8 µM nicotinamide and 111 mM glucose. The flasks were incubated at 30 ºC at a speed of 250 rpm for 15 hours. The mycelia were harvested by filtering through nylon gauze, dried briefly between paper towels and were then frozen in liquid nitrogen. The frozen mycelia of approximately 1 gram were grinded using a micro-dismembranator (B. Braun Biotech) and aliquots of 100-300 mg were transferred to 2 ml Eppendorf tubes which were then weighed to determine the approximate weight of the ground mycelia. These aliquots were then suspended in the appropriate extraction buffer at a concentration of 10 % wet weight. Mops buffer of pH 7.2 containing 10 % glycerol and 1 mM dithiothreitol and were used as extraction buffer for *ldh*A and *ldh*B transformants, whereas 0.1 M pH 7 potassium phosphate buffer containing 1 mM $MgCl_2$, 1.4 mM β-mercaptoethanol, 1 mM PMSF (phenylmethyl sulfonylflouride) and 0.1 mM TPP (thiamine pyrophosphate) was used for *pdc*A and *pdc*B transformants.

Lactate dehydrogenase activities were determined according to a spectrophotometric assay which measures decrease in NADH concentration at 340 nm [11]. The principle of the assay is shown in Figure 2.2.

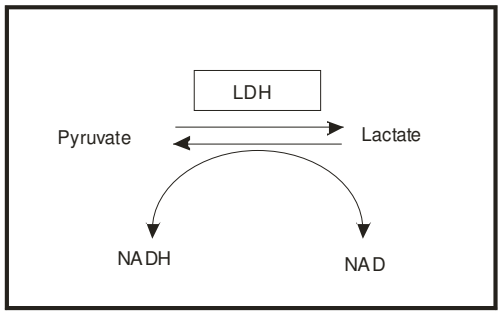

Figure 2.2: Principle of lactate dehydrogenase enzyme assay

The assay mixture was composed of 175 µM NADH, 4 mM sodium pyruvate and appropriately diluted crude extract in 0.1 M pH 6.8 Mes buffer. One unit of enzyme activity is defined as the amount of enzyme, which converts 1 µmole of NADH to 1 µmole NAD^+ in 1 minute at 30 ºC at pH 6.8.

Pyruvate decarboxylase activities were determined as described in section 2.1.5

Enzyme activities were measured by an automatised procedure which was performed by Cobas Bioanalyser (Roche Diagnostics). The activities were calculated using the extinction coefficient (ε) of NADH: 6.22 x 10^3 mM^{-1} cm^{-1}.

2.2.2.2.4.3. Analysis of organic acids produced by *Aspergillus niger* NW219 transformed with *ldh*A and *ldh*B of *Rhizopus oryzae*

In order to determine whether the *A. niger* NW219 transformed with *ldh*A and *ldh*B of *R. oryzae* the transformants are producing lactate, the transformants having higher lactate dehydrogenase specific activities compared to wild type were further analysed. Four *ldh*A transformants, 5 *ldh*B transformants and wild type were cultivated for 22 hours. For each transformants an amount of 10^6 spores/ml were inoculated into 250 ml flasks containing 70 ml minimal medium (Appendix D)

supplied with 0.1 % casamino acids, 0.1 % yeast extract, Vishniac solution (1 ml in 1 L), 1.5 mM leucine, 8 µM nicotinamide and 111 mM glucose. Five mM uridine was supplied into the medium of wild type. Samples of 1 ml from the media were taken at 12, 14, 16, 18, 20, 22 hours of growth and immediately frozen in liquid nitrogen and stored at -20 ºC until analysis.

The samples were analysed for 5 organic acids (lactate, citrate, oxalate, gluconate and acetate). Analyses were done by HPLC using an Aminex HPX-87H (Bio-Rad) column eluted with 25 mM HCl at 50 ºC and using UV (210 nm) detection.

2.3. Genomic library construction

2.3.1. Isolation of genomic DNA from *Rhizopus oryzae*

Genomic DNA was isolated from *R. oryzae* using a method based on the use of cethyltrietyl ammonium bromide (CTAB) [99]. Modifications were done on the original protocol to decrease the amount of degradation with endogenous nucleases. The modified DNA isolation protocol is given in section 2.2.1.1. and the compositions of the solutions are given in Appendix G.

2.2.3.2. Partial digestion of *Rhizopus oryzae* DNA with *Sau*3A and isolation of DNA fragments after agarose gel electrophoresis

The DNA (50 µg) isolated from *R. oryzae* ATCC 9363 as described in part 2.2.1.1 was partially digested by incubation of the DNA with 0.025 U *Sau*3A for 4 hours at 37 ˚C. The resulting fragments were size fractionated by electrophoresis on 0.6 % agarose gel in TAE buffer containing 0.5 µg/ml ethidium bromide. Fragments with a size between 14 kb to 22 kb were compared to fragments of bacteriophage λ DNA digested with *Bgl*II (22.0 13.3, 9.7, 0.65 and 0.44 kb) and recovered from the gel by cutting the appropriate region from the gel. As a control 10 µg of uncut

lambda DNA were processed in the same way for *R. oryzae* fragments. These fragments were recovered from the piece of agarose by electroelution using ISCO cups (Figure 2.3).

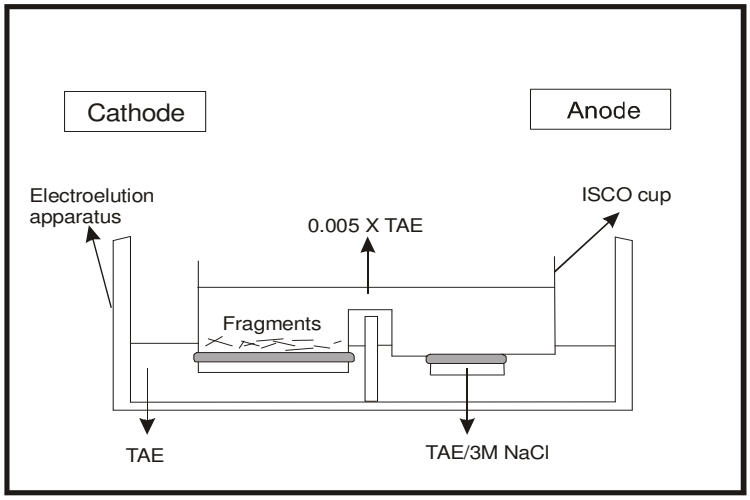

Figure 2.3: A scheme of the apparatus for electroelution with ISCO cups

A dialysis membrane was mounted on both the large and the small containers of this cup, the cup was filled with 0.005 x TAE (diluted from 50 x TAE stock solution (per 1000 ml): 242.0 g Tris; 57.1 ml glacial acetic acid; 100 ml 0.5 M

EDTA; adjusted to pH 8.0 with HCl) and the piece of agarose was placed in the large container of the cup. Subsequently, the cup was placed in the electroelution apparatus, with the large container in the cathode chamber containing TAE and the small container at the anode chamber containing TAE/3M NaCl (Figure 2.1. The fragments were electro-eluted at 100 V for a period of 2 hours. Afterwards, the cup was taken from the electroelution apparatus and the buffer was removed from the large container, while the buffer was removed only from the upper part of the small container. The remaining buffer (200 µl) containing the DNA fragments was dialysed in the cup against distilled water for a period of 30 minutes. Finally the DNA was precipitated by the addition of 0.1 volume 3 M NaAc, pH 5.6 and 2 volumes cold (-20 ºC) absolute ethanol. The DNA was collected by centrifugation (Eppendorf) for 30 minutes at 14 000 g at 4 ˚C. After removal of the supernatant, the DNA pellet was air-dried. Following ethanol precipitation, the DNA was dissolved in 10 µl TE buffer

(10 mM Tris-HCl pH 8.0; 1 mM EDTA pH 8.0) and the concentration was determined by agarose gel electrophoresis, using lambda DNA with a known concentration as a reference.

Alternatively, size fractionation of partially digested DNA was carried out on 0.6 % LMP (low melting point) agarose. Size fractionation is done as described before for agarose gel electrophoresis. The fragments recovered by cutting from the gel were then heated up to 45 ˚C, at which temperature the LMP-agarose melts. Once the gel is completely melted, phenol/chloroform extraction was done to recover the DNA from this solution. An equal volume of phenol is added to the melted agarose, after mixing well by inversions an equal volume of chloroform was added and mixed thoroughly, the mixture was centrifuged for 20 minutes at 10 000 g. The aqueous phase was extracted once more with phenol/chloroform (1:1) and was then extracted once with chloroform. The DNA was isolated from the aqueous phase by ethanol precipitation as described previously. The DNA fragments obtained were analysed by agarose gel electrophoresis, using 0.025 µg and 0.125 µg lambda DNA as references for concentration calculation.

2.2.4. Cloning genes from *Rhizopus oryzae*

As the attempts to construct a genomic library from *R. oryzae* have failed to give good results, a different method to clone genes for which the full sequence is not available, was considered necessary. This method is a PCR based technique, where DNA fragments can be amplified from the known into the unknown DNA sequence. These experiments were performed with the GenomeWalker Kit (Clontech). The method was evaluated for its use in cloning a part of hexokinase and glucokinase genes from *R. oryzae*.

2.2.4.1. Outline of the method

The method starts with construction of 'libraries'. The genomic DNA is completely digested by 4 different blunt-end cutter enzymes. The adapters are ligated to these fragments and the batches of fragments digested with adapters are called 'libraries'. Once the libraries are ready the fragment(s) of the gene of interest are generated in two PCR steps. The PCR products of the primary PCR are diluted 50X and used as a template for the nested PCR. The primers used in these PCR reactions, are Adapter Primer 1, Adapter Primer 2, Gene Specific Primer 1 and Gene Specific Primer 2 abbreviated as AP1, AP2, GSP1 and GSP2 in Figure 2.4 respectively. The outline of the method is given in Figure 2.4.

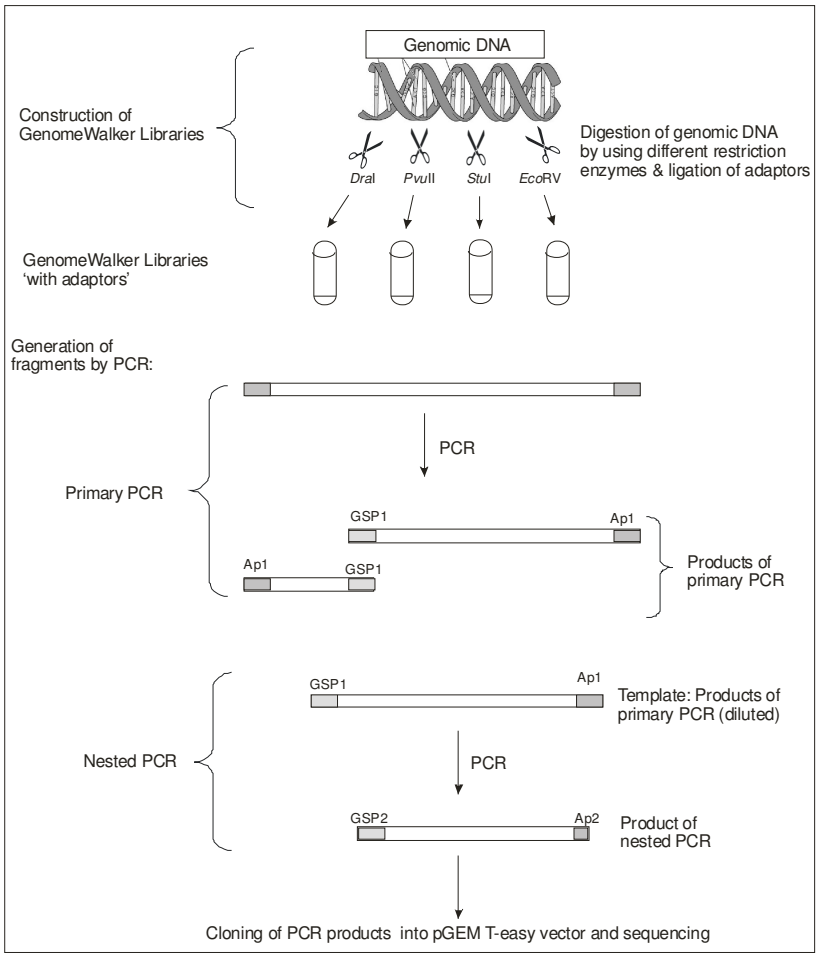

Figure 2.4: Schematic representation of the GenomeWalker kit principle

The kit supplies AP1 and AP2, which were designed based on the adapter sequence. Depending on the known sequence of the gene of interest GSP1 and GSP2 are designed. Genome Walker reactions are performed with the Advantage Genomic polymerase mix (Clontech) containing DNA polymerases suitable for long-distance PCR [100, 101]. The polymerase mix contains a *Tth* DNA polymerase as the primary polymerase and a minor amount of a second DNA polymerase, *Vent*R, to provide 3'→5' exonuclease activity. The TthStart™ Antibody is included to provide automatic "hot start" PCR. Furthermore the 3' A-tailing function of *Tth* polymerase allows to clone the PCR products easily by using TA cloning strategy into pGEM-T® easy vector. The fragments obtained at the end of two successive PCR are then cloned and characterised.

2.2.4.2. Constructing 'libraries'

Genomic DNA was isolated from *R. oryzae* mycelium using a method based on the use of cethyltrimethyl ammonium bromide (CTAB) [99]. Since the construction of GenomeWalker libraries should start with clean and high molecular weight DNA, the isolated DNA was further analysed for its quality. In order to check the molecular weight (MW) of the DNA 1 µl (0.1 µg) of *R. oryzae* genomic DNA and 1 µl (0.1 µg) of control genomic DNA which is included in the kit was run on a 0.5 % agarose gel. To check the purity the 0.5 µg of genomic DNA were digested with 16 units of *Dra*I in a total volume of 20 µl. A control without enzyme was set up and digestion was carried out overnight (o/n) at 37 ˚C. Five µl of each reaction was analysed on a 0.5 % agarose gel along with 0.5 µl of experimental genomic DNA as a control. The concentration of DNA was approximately 0.1 µg/µl.

For library construction a total of five reactions were done; four digestion of *R. oryzae* genomic DNA by four different restriction enzymes; *Dra*I, *Pvu*II, *Stu*I and *Eco*RV and one *Pvu*II digestion of human genomic DNA as a positive control. Thirty five µl (3.5 µg) of DNA was digested for each restriction enzyme. After analysis of the digested DNA samples, the DNA was purified by phenol/chloroform extraction

and ethanol precipitation. The pellets obtained after ethanol precipitation were dissolved in 20 µl of TE buffer (10 mM Tris HCl 0.1 mM EDTA, pH 7.5). The approximate quantity of the DNA was determined by running 1 µl of the mixture on a 0.5 % agarose gel. Then, the ligation reactions of adapters and the DNA fragments were set done: 1.9 µl GenomeWalker adapter (25 µM), 1.6 µl 10x ligation buffer, 0.5µl T4 DNA ligase (6 U/µl) and 4 µl digested, purified DNA. The reaction mixtures were incubated at 16 ˚C overnight in a PCR thermocycler. The reactions were stopped by incubating at 70 ˚C for 5 minutes. Finally 72 µl of TE (10 mM Tris HCl, 1 mM EDTA, pH 7.5) was added to each tube containing the ligation mixtures. For convenience these DNA fragments with adapters will be referred as 'libraries'.

2.2.4.3. Primers used for generating fragments

Adapter Primer 1 (AP1) and Adapter Primer 2 (AP2) were supplied in the kit and gene specific primer 1 and gene specific primer 2 were designed on the basis of the known *hxk* and *glk* sequences of closely related organisms. For hexokinase gene specific primer, *hxk* sequences from different fungi have been aligned by ClustalW tool from the NCBI web page and the sequences, which are less alike with the others, have been removed from the alignment. Finally the alignment of *hxk* sequences from *Kluyveromyces lactis*, *Aspergillus niger*, *Debaromyces occidentalis* and *Saccharomyces cerevisiae* were used for designing the degenerate primers for hexokinase. Degenerate primers for glucokinase gene have been designed in the same way, gene sequences from *A. niger*, *S. cerevisiae* and *Pichia angusta* have been used**.** The primers were designed so that their melting temperatures are close to 67 ˚C, which is the recommended temperature for the annealing and extension reactions. The GSP1 and GSP2 sequences for hexokinase were based on the 1428-1453 bp and 1088-1117 bp regions of *A. niger hxk*. The regions corresponding to 1677-1703 bp and 2068-2104 bp of *A. niger glk* sequence were used for designing the GSP1 and GSP2 for *glk*.

2.2.4.4. Walking along the genomic DNA

Amplification of the unknown DNA fragments was done with two subsequent PCR's. First, a primary PCR was performed using the adapter primer1 (AP1) and gene specific primer 1 for *hxk* or *glk* in the 4 different libraries. After 1/50 dilution of the primary PCR products, nested PCR was done using adapter primer 2 (AP2) and a gene specific primer 2 for *hxk* or *glk* for each of the library products. The PCR conditions for primary and nested PCR given in Table 2.3.

Table 2.3: The PCR conditions for primary and nested PCR of GenomeWalker kit

NUMBER OF CYCLES	TEMPERATURE	TIME
7	94 ˚C	2 seconds
	70 ˚C	3 minutes
37	94 ˚C	2 seconds
	65 ˚C	3 minutes
1	67 ˚C	4 minutes
	4 ˚C	pause

After the nested PCR is complete, 8 µl from each of the 50 µl PCR reaction mixtures were run on the gel to check for the product formation. After examining the products on agarose gel, the fragments were ligated into the pGEM®-T easy vector using a kit, which make use of TA cloning strategy (Promega pGEM®-T easy vector systems). Ligation was performed in a total of 10 µl reaction mixture composed of 5 µl of 2 X rapid ligation buffer, 1 µl (50 ng) of vector, 3 µl from nested PCR product and 1 µl T4 DNA ligase. Ligation was performed over night at 16 ˚C. Five µl of this ligation mixture was used for transformation of *E. coli* DH5α cells (Appendix H). Ten white and pale blue colonies were picked from the transformation plates of each library for minipreps. The plasmids isolated from these colonies were tested for presence of an insert by digesting 2 µl (0.1-0.5 µg DNA) from each with *Not*I

enzyme and run on a 0.8 % agarose gel. The ones, which contain inserts, were sequenced by GATC-reaction mix (Amersham Pharmacia Biotech) using SP6 and T7 primers.

2.2.4.5. Analysis of the sequences

2.2.4.5.1. Blast search of the sequences obtained by GenomeWalker

Analysis of product sequences was performed using several tools of bioinformatics. After removing the vector and anchor sequences, the amplified fragment sequences were compared to the non-redundant GenBank [102] databases using the tblastx and blastx alignment programs. The tblastx tool searches for the similarity between translated sequence and translated database sequences, while blastx searches for the similarity between the translated sequence and protein sequences within the database.

2.2.4.5.2. Southern blot analysis

In order to check if there were any hexokinase sequence(s) in the GenomeWalker primary and nested PCR products, Southern blot analysis was performed using a heterologous hybridisation strategy. Eight µl from each of the reactions were run o/n at 18 V on 0.8 % agarose (in TAE buffer containing 0.5 µg/ml ethidiumbromide) along with 0.5 µg of the *HinD*III-digested lambda DNA marker. The protocol for southern blot and hybridisation is given in Appendix L. The hexokinase gene (*hxk*) (AJ009973) from *A. niger* was used as a heterologous probe for the hybridisation.

In order to determine the gene copy number of *hxk* and *glk* in *R. oryzae* Southern blot was performed using *A. niger hxk* and *glk* sequences as probes for heterologous hybridisation. Two µg of *R. oryzae* genomic DNA were digested by using 20 U of *Eco*RI. After incubation at 37 ˚C o/n, the digested DNA was run o/n at

18 V on 0.8 % agarose (in TAE buffer containing 0.5 µg/ml ethidiumbromide) along with 0.5 µg *Eco*RI/*Hin*DIII-digested lambda DNA and 0.5 µg *Hin*DIII-digested lambda DNA marker. The southern blot analysis was performed as described in Appendix L. The hexokinase (Accession Number: AJ009973) and glucokinase gene (AN: X99626) sequences of *A.niger* were used as heterologous probes.

The probes were labelled radioactively with [α-^{32}P]dATP using megaprime DNA labelling system (Amersham Pharmacia) according to manufacturer instruction. Twenty five nanogram of template DNA (5 µl)was labelled by addition of 5 µl (3000 Ci/mmol) [α-^{32}P] dATP in a volume of 50 µl. Labelling was carried out at 37 °C for 30 minutes. No further purification of the probe was necessary and it was used directly by addition to the hybridisation medium (Appendix L). Once the hybridisation is complete the membranes were placed in a cassette. The film was exposed for one week and was developed.

CHAPTER 3

RESULTS

3.1. Purification and characterisation of pyruvate decarboxylase from *Rhizopus oryzae*

3.1.1. Purification of pyruvate decarboxylase

Pyruvate Decarboxylase was purified from *Rhizopus oryzae* up to 33 fold with a 32 % yield in three steps composed of ammonium sulfate fractionation, gel filtration column chromatography and DEAE-cellulose column chromatography (Table 3.1).

Table 3.1: Purification table of pyruvate decarboxylase from *Rhizopus oryzae*

Purification step	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Total Activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	38	12.98	4.59	494	174.4	2.83	1	100
$(NH_4)_2SO_4$ precipitation	10	62.80	4.52	628	45.2	13.89	4.9	127
Gel filtration (G 150)	21	21.70	0.60	456	12.6	36.11	12.8	92
Anion-exchange	9	17.72	0.19	160	1.7	94.12	33.3	32

In elution profile of gel filtration column chromatography (Figure 3.1) you can see that the activity is eluted in a single peak.

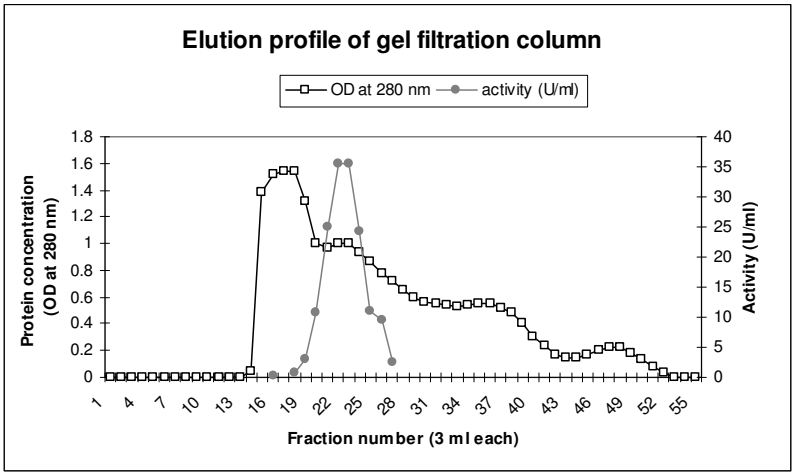

Figure 3.1: Elution profile from gel filtration column chromatography. Sephadex G 150 was used in the column of 2.2x35 cm dimensions.

From the anion exchange column chromatography the activity peak of the PDC was eluted as a single peak after a relatively bigger peak most probably containing the bulk of proteins.

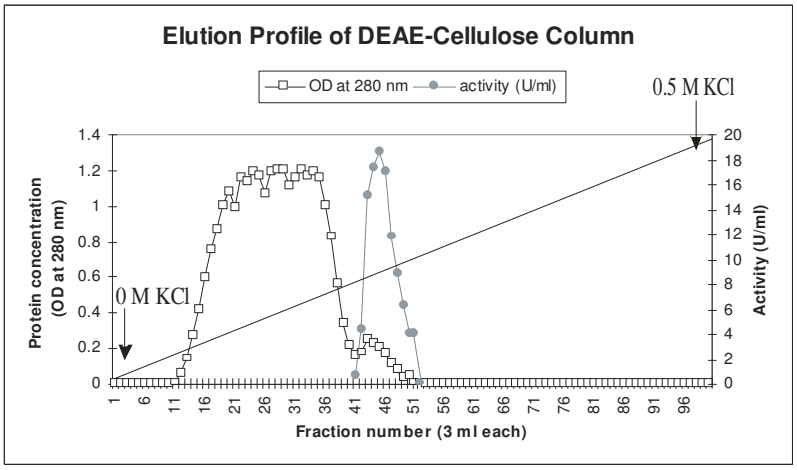

Figure 3.2: Elution profile of DEAE-cellulose column chromatography. The straight diagonal show the linear gradient of KCl applied. The start and the end of the gradient is shown with an arrow and the concentration is indicated as molatiry above the arrows.

SDS-PAGE analyses of samples taken from different steps of purification are shown in Figure 3.3.

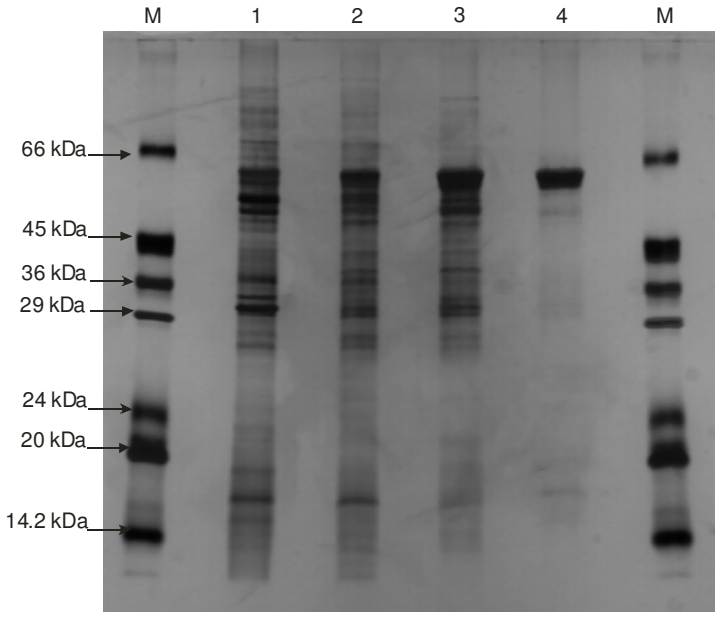

Figure 3.3: SDS-PAGE analysis of samples from different steps of purification. Molecular weight markers are marked as 'M' above the lanes, crude extract (lane 1), 35-55 % ammonium sulphate fraction after dialysis (lane 2), pooled fractions of gel filtration column (lane 3), sample from DEAE-cellulose column chromatography (lane 4).

The amount of protein loaded to each well was 10 µg except for the samples of DEAE-cellulose column, which were 5 µg each. As it can be seen from the figure (Figure 3.3) at each step of purification the number of bands was decreased compared to the previous step and the major band obtained at the end of the last step of purification could be visualised throughout the whole purification procedure.

3.1.2. Molecular weight determination of pyruvate decarboxylase

Monomer molecular weight of the purified PDC was calculated as 59±2 kDa by SDS PAGE analysis (Figure 3.3).

3.1.3. Activity staining of pyruvate decarboxylase

Activity staining results suggested that the major band in the partially purified sample showed PDC activity. PDC activity was visualised as white precipitate formed as a result of condensation of acetaldehyde by binding to the dianilinoethane.

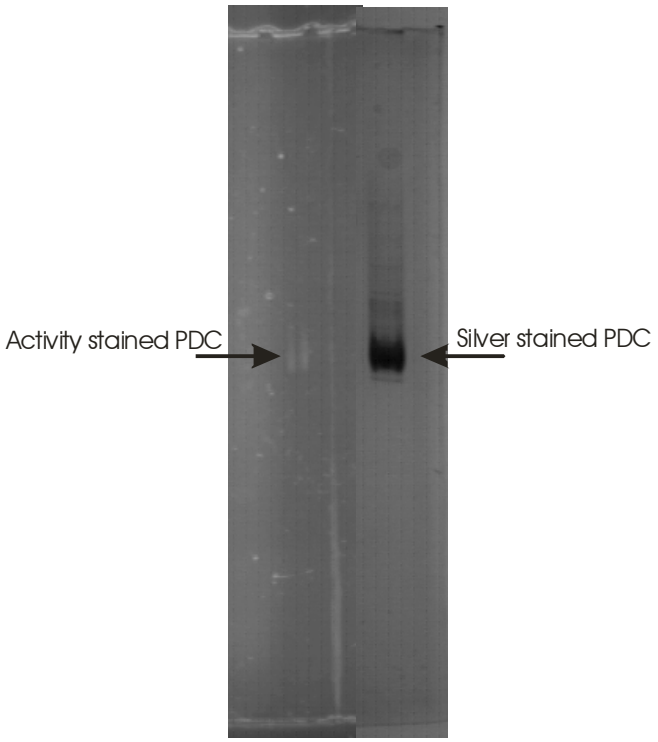

Figure 3.4: Native PAGE (5-22.5 %) analysis of the activity stained and silver stained purified pyruvate decarboxylase (PDC) from *R. oryzae*.

3.1.4. Kinetic analyses of PDC

The kinetic analyses of the enzyme showed that the velocity curve of the enzyme does not follow the normal Michaelis-Menten kinetics. The velocity curve is slightly sigmoidal, which is seen more clearly when the velocity (υ) versus substrate concentration ([S]) curve was drawn at pyruvate concentration between 0.1 and 30 mM (Figure 3.5).

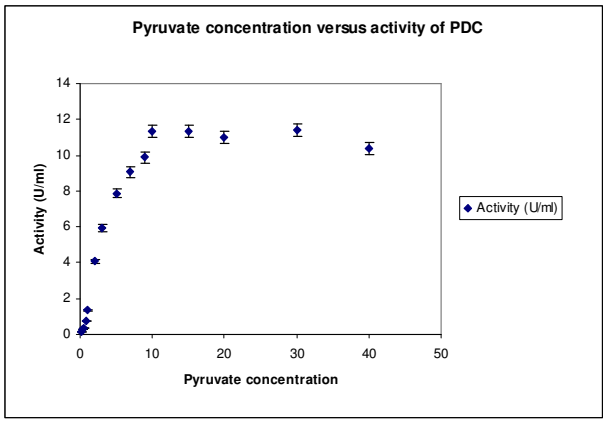

Figure 3.5: Kinetics of purified pyruvate decarboxylase for pyruvate (0.1-30 mM). The reactions were measured at 35 °C at pH 6.5 in a mixture containing 0.2 mM thiamine pyrophosphate, 5 mM $MgCl_2$, 0.15 mM NADH. The concentration of the enzyme in the reaction was approximately 0.01 mg/ml.

The kinetic data at different pyruvate concentrations were analysed by means of the Hill Equation and Eadie-Scatchard Plot for allosteric enzymes. The velocity data for pyruvate concentration between 0.5-30 mM were used for calculation of K_m for the PDC. The K_m was calculated as 8.6 mM from 1/υ versus $1/[S]^2$ graph (Figure 3.6).

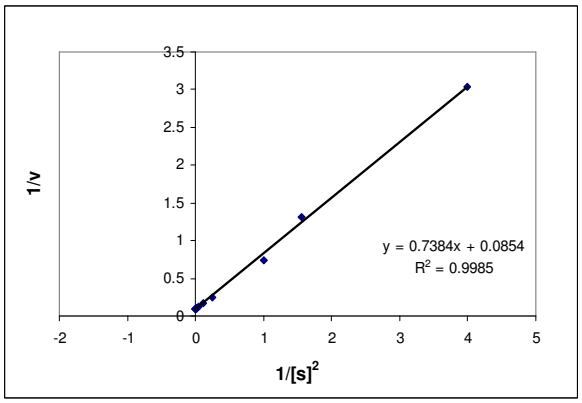

Figure 3.6: 1/υ versus $1/[S]^2$ graph of the purified pyruvate decarboxylase

The enzyme is inhibited at pyruvate concentrations higher than 30 mM. The remaining activity was 65 % of the maximum activity at pyruvate concentration of

70 mM and only 6 % of the maximum activity was present at a pyruvate concentration of 150 mM (Figure 3.7).

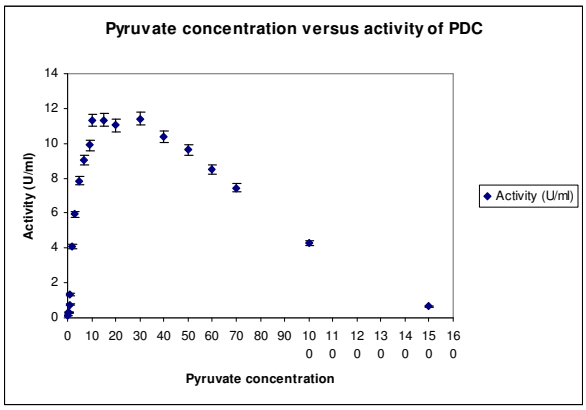

Figure 3.7: Kinetics of purified pyruvate decarboxylase for pyruvate (0.1-150 mM). The reactions were measured at 35 °C at pH 6.5 in a mixture containing 0.2 mM thiamine pyrophosphate, 5 mM MgCl2, 0.15 mM NADH. The concentration of the enzyme in the reaction was approximately 0.01 mg/ml.

The Hill coefficient (n_H) was calculated as 1.8 as the slope of the linear portion of the Hill Plot (Figure3.8), which indicates that PDC from *R. oryzae* exhibits co-operative binding.

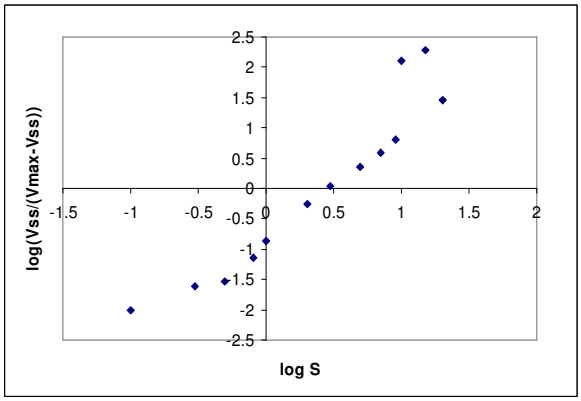

Figure 3.8: Hill plot of purified pyruvate decarboxylase

The pH dependence of the PDC was analysed in a pH range of 5.5 and 8.0 (Figure 3.9).

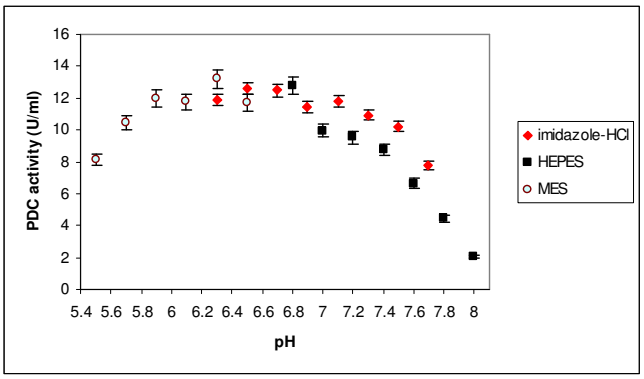

Figure 3.9: pH dependence of pyruvate decarboxylase activity. The reactions were measured at 35 °C at varying pH values in a mixture containing 25 mM sodium pyruvate, 0.2 mM thiamine pyrophosphate, 5 mM $MgCl_2$, 0.15 mM NADH. The concentration of the enzyme in the reaction was approximately 0.01 mg/ml.

The enzyme showed its maximum activity (V_{max}) at pH 6.5. The pH dependence of the enzyme was not very sharp. The enzyme had an activity of 92-100 % of the V_{max} between pH 5.9 and 7.1. At slightly alkaline pH's (7.6-8.0) the decrease in enzyme activity declined at a higher rate, at pH 8.0 the remaining activity was only 16 % of the V_{max}.

3.2. PCR cloning of lactate dehydrogenase A (*ldh*A), lactate dehydrogenase B (*ldh*B), pyruvate decarboxylase A (*pdc*A) and pyruvate decarboxylase B (*pdc*B)of *Rhizopus oryz*ae and transformation of *Aspergillus niger* with these genes

3.2.1. PCR cloning of *ldh*A, *ldh*B, *pdc*A and *pdc*B from *Rhizopus oryzae*

Four genes of *Rhizopus oryzae* (*ldh*A, *ldh*B, *pdc*A and *pdc*B) were cloned successfully and as determined by the sequences of these clones there were no mutations changing the sequence of their translated products.

3.2.2. Analysis of *Aspergillus niger* NW219 transformed with *ldh*A, *ldh*B of *Rhizopus oryzae*

A total of 22 colonies for ldhA transformants and 30 colonies for ldhB transformants were collected from the transformation plates and plated twice on complete medium plates.

3.2.2.1. PCR screening of *Aspergillus niger* NW219 transformed with *ldh*A and *ldh*B of *R. oryzae*

Out of 22 ldhA transformants 13 of them contained the PCR product of expected size which is given in Table 2.1 (Figure 3.10). For the ldhB transformants there were 12 PCR positive transformants out of 30 (Figure 3.11). Thus co-transformation frequency for the ldhA transformants is 59 % while that of ldhB transformants is 40 %.

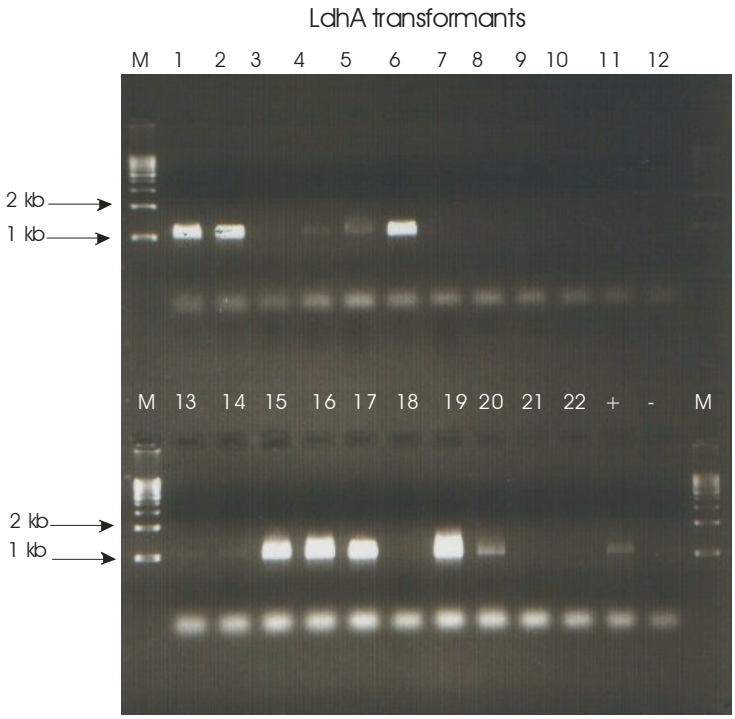

Figure 3.10: The agarose gel photo showing the PCR screening results for ldhA transformants.

In figure 3.10 the identity numbers of the transformants are given above each well. ‘M’ stands for molecular weight marker while ‘+’ indicates the positive control with *R. oryzae* DNA and ‘-‘ indicates the negative control with *A. niger* DNA. The bands corresponding to 1 kb and 2 kb of molecular weight are indicated by arrows. The expected size of the band for PCR products for *ldh*A transformants is 1.1 kb.

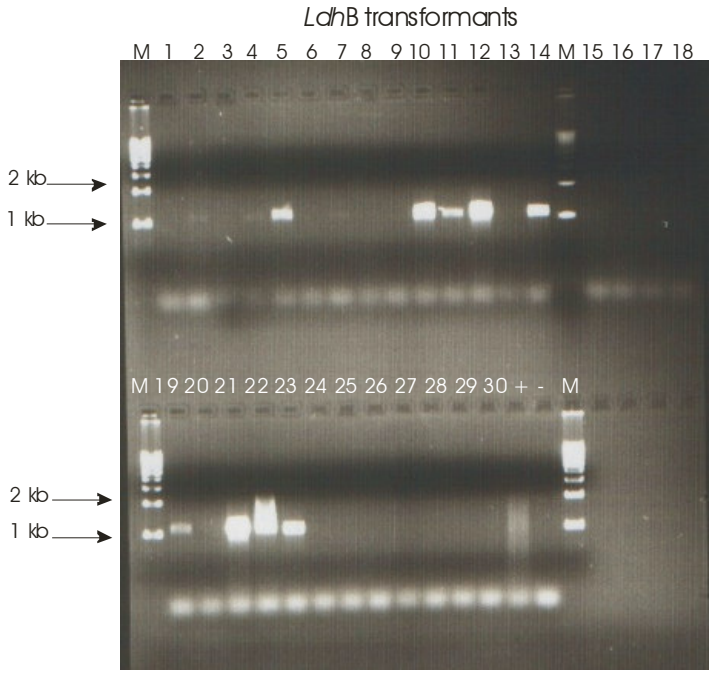

Figure 3.11: The agarose gel photo showing the PCR screening results for *ldh*B transformants.

In figure 3.11 the identity numbers of the transformants are given above each well. ‘M’ stands for molecular weight marker while ‘+’ indicates the positive control with *R. oryzae* DNA and ‘-‘ indicates the negative control with *A. niger* DNA. The bands corresponding to 1 kb and 2 kb of molecular weight are indicated by arrows. The expected size of the band for PCR products for *ldh*B transformants is 1.1 kb.

3.2.2.2. Enzyme activities of *Aspergillus niger* NW219 transformed with *ldh*A and *ldh*B of *R. oryzae*

Some of the transformants were shown to have higher specific activities compared to wild type, whereas others have even lower specific activity (Table 3.2).

Table 3.2: Lactate dehydrogenase activities of *ldh*A, *ldh*B transformants and wild type. Numbers were given to indicate the identity. The ones which are having higher specific activity compared to wild type are shown in bold character.

sample name	Activity (U/ml)	Protein (mg/ml)	Specific activity (SA)(U/mg protein)	Fold (SA/SA of wild type)
Wild type	1.6	3.0	0.53	1
ldhA1	0.7	2.8	0.25	0.47
ldhA2	1.3	2.9	0.45	0.85
ldhA4	**3.3**	**3.3**	**1.00**	**1.90**
ldhA5	1.3	2.7	0.48	0.91
ldhA6	**2.3**	**3.4**	**0.68**	**1.28**
ldhA13	0.3	1.7	0.18	0.34
ldhA14	**2.5**	**3.9**	**0.64**	**1.21**
ldhA15	**1.9**	**3.2**	**0.59**	**1.11**
ldhA16	1.0	2.7	0.37	0.70
ldhA17	1.6	4.1	0.39	0.74
ldhA19	0.8	2.8	0.29	0.55
ldhA20	1.4	3.0	0.47	0.89
ldhA22	1.4	2.6	0.54	1.02
ldhB2	1.3	2.6	0.50	0.94
ldhB4	0.9	3.0	0.30	0.57
ldhB5	**3.2**	**3.7**	**0.87**	**1.23**
ldhB7	1.3	2.5	0.52	0.98
ldhB10	**3.5**	**2.8**	**1.25**	**2.36**
ldhB11	1.2	2.6	0.46	0.87
ldhB12	**17**	**3.2**	**5.31**	**10.0**
ldhB14	1.8	3.2	0.56	1.06
ldhB19	1.4	2.9	0.48	0.91
ldhB20	**6.1**	**3.1**	**1.97**	**2.77**
ldhB22	**3.2**	**3.1**	**1.03**	**1.94**
ldhB23	1.9	3.0	0.63	1.19

Table 3.2 gives the protein and enzyme activity data and the fold of difference of specific activity between the transformants and the wild type. There were 4 *ldh*A transformants and 5 *ldh*B transformants having higher specific activities compared to wild type. The difference between the specific activities of wild type and transformants varied among the transformants, the most significant is for *ldh*B12 transformant which has a 10 fold higher specific activity than wild type. The highest specific activity among the *ldh*A transformants was 2 fold for *ldh*A4. Some of the transformants did not have a significantly higher fold, whereas the others had even lower specific activity values than wild type.

3.2.2.3. Organic acids production by *Aspergillus niger* NW219 transformed with *ldh*A and *ldh*B of *R. oryzae*

Production of organic acids was determined for the *ldh*A and *ldh*B transformants which was chosen depending on the enzyme activity analysis. The graph showing the lactic acid production in the *ldh*A and *ldh*B transformants of *A. niger* is given in Figure 3.12 which is the graphical form of the data given in Table 3.3.

Table 3.3: Lactate production rates of wild type and transformants

	lactate produced (mM)									
time (hours)	Wild type	ldhA4	ldhA6	ldhA14	ldhA15	ldhB5	ldhB10	ldhB12	ldhB20	ldhB22
12	0.88	2.97	2.37	2.69	3.05	3.17	3.45	2.67	2.84	2.61
14	1.15	4.09	3.03	3.53	3.91	3.73	4.51	3.83	3.94	3.70
16	1.56	4.70	3.93	4.36	5.01	4.07	5.08	4.27	4.44	5.07
18	1.91	4.78	3.73	4.04	5.09	4.73	5.74	4.67	5.11	4.78
20	2.23	4.67	4.00	4.56	4.80	4.85	5.92	4.91	5.54	5.04
22	2.65	4.65	4.29	4.17	5.22	4.54	4.51	6.37	5.31	4.34

All of the *ldh*A and *ldh*B transformants analysed for lactate production appeared to produce higher amount of lactate compared to wild type under the conditions tested. As can be visualised from the graphical representation of the data (Figure 3.12) the lactate production rate decreased by time. This can be followed as the decrease of the slope after 18 hours of incubation.

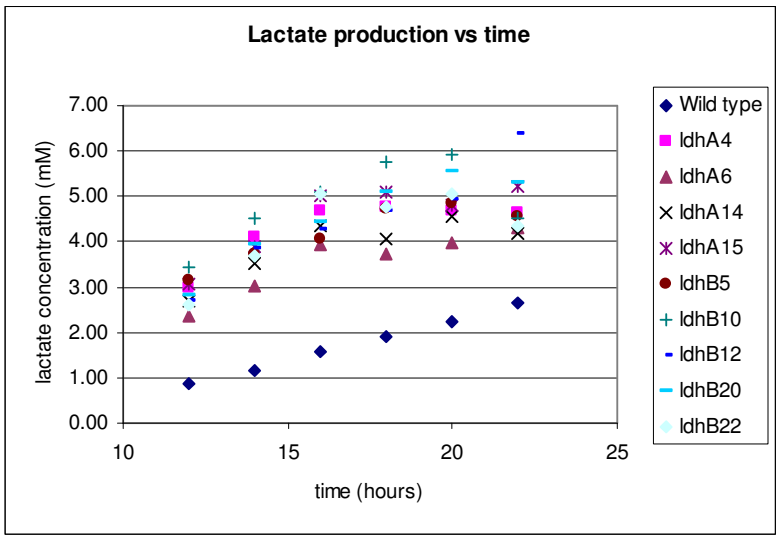

Figure 3.12: Graphical view of the lactate production of wild type and *ldh*A and *ldh*B transformants of *A. niger*

Aspergillus niger is a very well known citrate producer and the effect of the new branch (lactate production) introduced into this organism was observed as an increase in the citrate production of the transformants compared to the wild type (Table 3.4) under the conditions tested.

Table 3.4: Citrate production by wild type and transformants. The abbreviation 'ns' stands for 'not significant'.

	citrate produced (mM)									
time (hours)	**Wild type**	**ldhA4**	**ldhA6**	**ldhA14**	**ldhA15**	**ldhB5**	**ldhB10**	**ldhB12**	**ldhB20**	**ldhB22**
12	ns	0.20	0.17	0.14	ns	0.14	ns	0.18	ns	ns
14	ns	0.65	0.45	0.50	0.41	0.52	0.40	0.70	0.38	0.38
16	0.25	1.30	0.98	1.10	1.06	1.05	0.91	1.34	1.02	1.04
18	0.56	2.14	1.63	1.69	1.93	1.98	1.78	2.29	2.09	2.07
20	0.93	2.76	2.18	2.53	2.56	2.68	2.56	3.12	3.15	3.02
22	1.49	3.61	3.00	3.08	3.57	3.04	3.66	3.52	4.00	3.58

As indicated in table 3.4 all the transformants showed an increased amount of citric acid production. The concentration of citrate produced at the end of 22 hours of incubation was 1.5 mM for wild type, whereas for transformants these values were higher than this value and the lowest being 3 mM. The increased rate of citrate production can be visualised better in Figure 3.13.

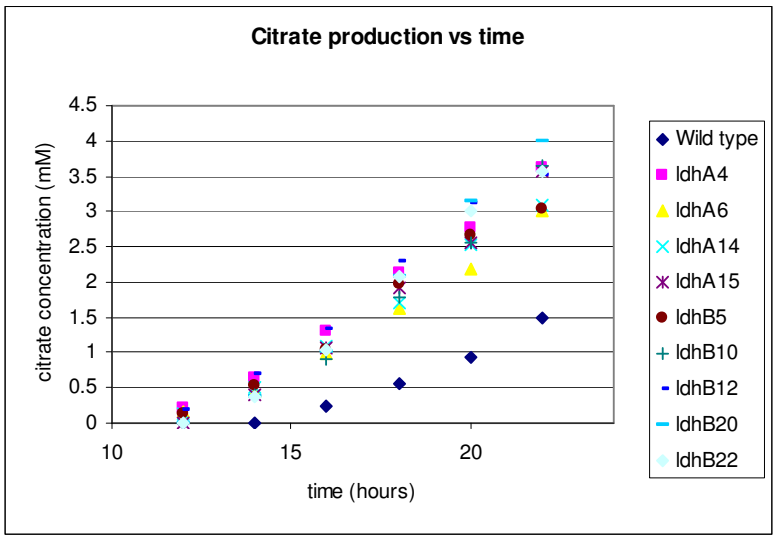

Figure 3.13: Graphical representation of citrate production of wild type and *ldh*A and *ldh*B transformants of *A. niger*

The rate of the increase in citrate production represented by the slopes of the lines were slightly higher than the rate of the wild type which can be seen from the graph (Figure 3.13). Moreover, the onset of citrate production is earlier for the transformants than wild type.

Another difference between the wild type and the transformants was that no gluconate production was detected in the media samples of the transformants.

3.2.3. Analysis of *Aspergillus niger* NW219 transformed with *pdc*A and *pdc*B of *Rhizopus oryzae*

A total of 52 colonies (22+30) were collected from the two transformation experiments where the *R. oryzae pdc*A was introduced into *A. niger* NW219. Sixteen colonies were collected from the *pdc*B transformation plates and plated twice on complete medium plates.

3.2.3.1. PCR screening of *Aspergillus niger* NW219 transformed with *pdc*A and *pdc*B of *R. oryzae*

Out of 22 colonies of *pdc*A transformants which were collected in the first transformation experiments 5 of them contained the PCR product of expected size which was calculated as ~2000 bp (Table 2.1). In the second transformation experiment this value decreased to single colony out of 30 colonies collected. The transformation frequency for the first transformation done with *pdc*A was 23 % whereas this value decreased to only 3 % in the second transformation experiment done with the same gene. For *pdc*B transformants the expected size of the PCR product was ~2000 bp (Table 2.1) and 2 (*pdc*B4 and *pdc*B5) out of 16 transformants were PCR positive. The transformation frequency for this gene was 12.5 %. There were PCR products of ~ 2500 bp in 10 of the *pdc*B transformants including the ones containing the 2000 bp product (Figure 3.14). All of the 10 transformants containing any PCR product were further analysed.

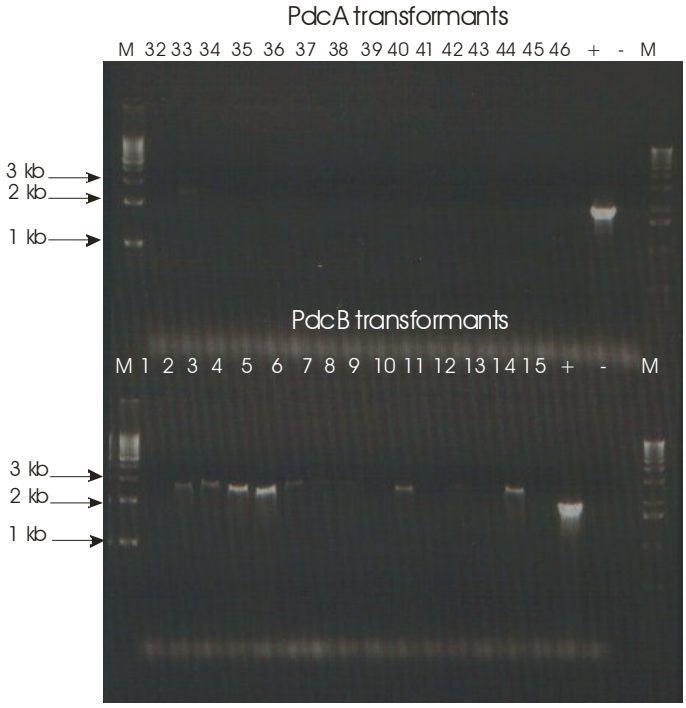

Figure 3.14: The agarose gel photo showing the PCR screening results for *pdc*A and *pdc*B transformants.

The identity numbers of the transformants are given above each well in figure 3.14. 'M' stands for molecular weight marker while '+' indicates the positive control run with *R. oryzae* DNA and '-' indicates the negative control run with *A. niger* DNA. The bands corresponding to 1 kb,2 kb and 3 kb of molecular weight are indicated by arrows. The expected size of the band for PCR products for *pdc*A and *pdc*B transformants is slightly higher than 2 kb.

the results for only15 of the 30 *pdc*A transformants are given for simplicity cause there are no products with the rest of the 15 picked from the transformation plates (Figure 3.14). These are the results of the second transformation experiment for *pdc*A where only the transformant number 33 contain the PCR product of expected size. For *pdc*B the PCR product of expected size (~2 kb) is present in transformant number 4 and 5 as a very faint band which can not be visualised from the photo because of the interference with the easily visualised of PCR product of 2.5 kb size.

3.2.3.2. Enzyme activities of *Aspergillus niger* NW219 transformed with *pdc*A and *pdc*B of *Rhizopus oryzae*

The pyruvate decarboxylase activities of the *pdc*A and *pdc*B transformants and wild type are given in Table 3.5.

Table 3.5: Pyruvate decarboxylase activities of *pdc*A, *pdc*B transformants and wild type. Numbers were given to indicate the identity. The ones which are having higher specific activity compared to wild type are shown in bold characters.

Sample name	Activity (U/ml)	Protein (mg/ml)	Specific activity (SA)(U/mg protein)	Fold (SA/SA of wild type)
Wild type	11.1	3.5	3.2	1
pdcA4	7.9	3.3	2.4	0.8
pdcA15	**19.3**	**4.1**	**4.7**	**1.5**
pdcA19	**7.1**	**2.8**	**2.5**	**1.3**
pdcA21	5.5	3.4	1.6	0.5
pdcA33	**15.0**	**3.5**	**4.3**	**1.3**
pdcB2	7.5	3	2.5	0.8
pdcB3	9.7	3.5	2.8	0.9
pdcB4	10.4	3.3	3.2	1.0
pdcB5	11.7	3.5	3.3	1.0
pdcB6	7.5	3.3	2.3	0.7
pdcB7	2.9	3	1.0	0.3
pdcB8	6.1	3.3	1.8	0.6
pdcB10	7.8	3.4	2.3	0.7
pdcB13	9.6	4	2.4	0.7
pdcB15	6.9	3.5	2.0	0.6

The analysis of the wild type, PCR positive *pdc*A and *pdc*B transformants for the pyruvate decarboxylase activity is summarized in Table 3.2. The table contains the protein and enzyme activity values, specific activities of the strains. The last column is composed the ratio of specific activity of the transformant over specific activity of the wild type. This table demonstrates that there are 3 *pdc*A transformants having higher specific activity than the wild type. However, none of the transformants chosen for *pdc*B showed higher specific activity than the wild type.

3.3. Genomic library construction

The protocol used for isolation of genomic DNA from *R. oryzae* was originally developed for bacteria. Some modifications were done to optimise the protocol for isolation of DNAse-free high molecular weight genomic DNA. For each small-scale isolation the starting material was determined as 150-200 mg ground mycelia in 900 µl of extraction buffer. The amount of SDS was increased from 1 mM

to 2 mM and of ProteinaseK from 0.1 µg/ml to 0.2 µg/ml. The quality of the DNA was evaluated by testing the digestibility of the DNA by *Sau*3A. To test that the isolated DNA is free of DNAses a control incubation in *Sau3*A buffer was done.

The fragments obtained after electroelution and ethanol precipitation seemed to be not dissolved. This was inferred from analysis of these fragments by agarose gel electrophoresis.

The samples resulting from electroelution and ethanol precipitation stayed in the wells whereas; the samples of DNA taken before electroelution showed a normal behaviour in the agarose gel electrophoresis.

3.4. Cloning genes from *Rhizopus oryzae*

GenomeWalker kit from Clontech was used to clone a part of hexokinase and glucokinase gene from *R. oryzae*. Degenerate primers were used since no sequence information was available for hexokinase or glucokinase in *R. oryzae*. The *hxk* sequences from *S. cerevisiae*, *K.lactis*, *D. occidentalis* and *A. niger* and *glk* sequences from *S. cerevisiae*, *P. angusta* and *A. niger* were aligned and the degenerated primers were designed in the highly conserved regions.

3.4.1. Primary and nested PCR products

The clustalW alignments which were used for the design of gene specific primers for hexokinase and glucokinase are shown in Figure 3.15 and 3.16.

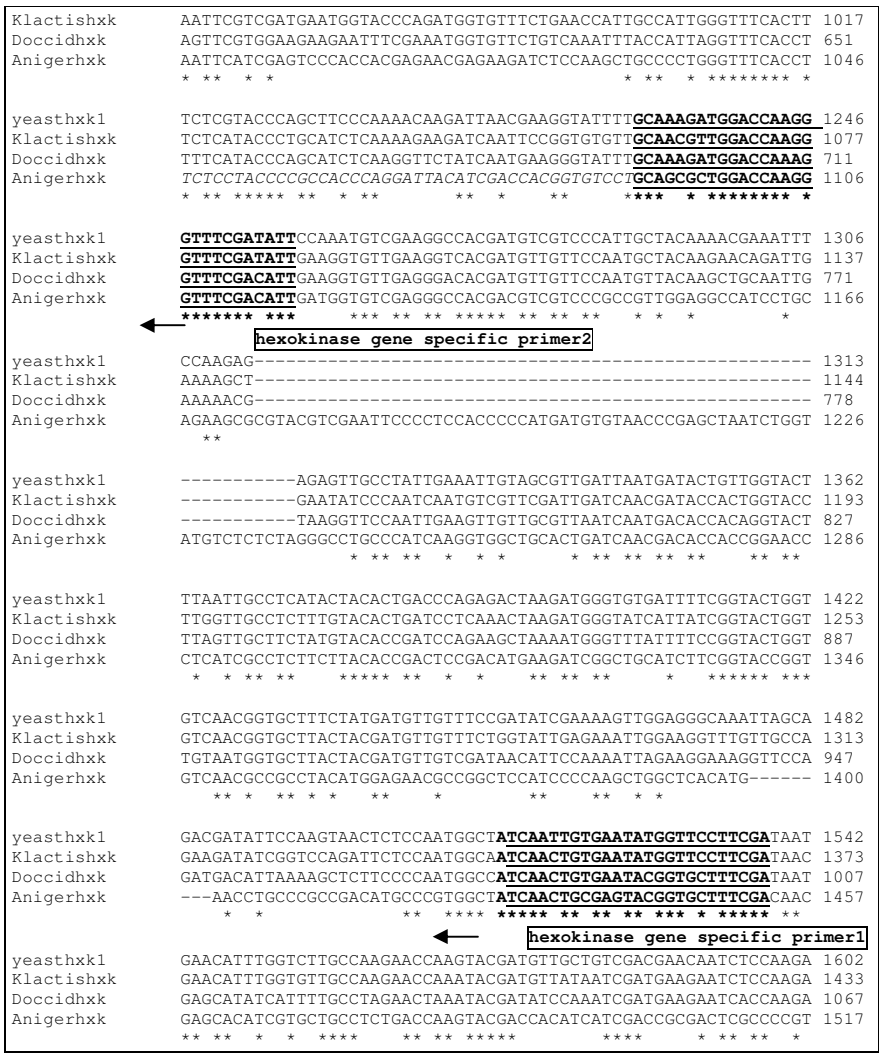

Figure 3.15: The ClustalW analysis of the gene sequences used for designing gene specific primer 1 and gene specific primer 2 for hexokinase. The sequence labels in the figure stand for: yeasthxk1; *S. cerevisiae* hexokinase1, Klactishxk; *K. lactis* hexokinase , Doccidhxk; *D. occidentalis* hexokinase, Anigerhxk; *A. niger* hexokinase. The figure shows only a part of the alignment where the primer sequences are highlighted by underlining and bold letters. The directions of the arrows are from 5' to 3' end of the primers and the names of the primers are given in the boxes above the regions that the primers are based on.

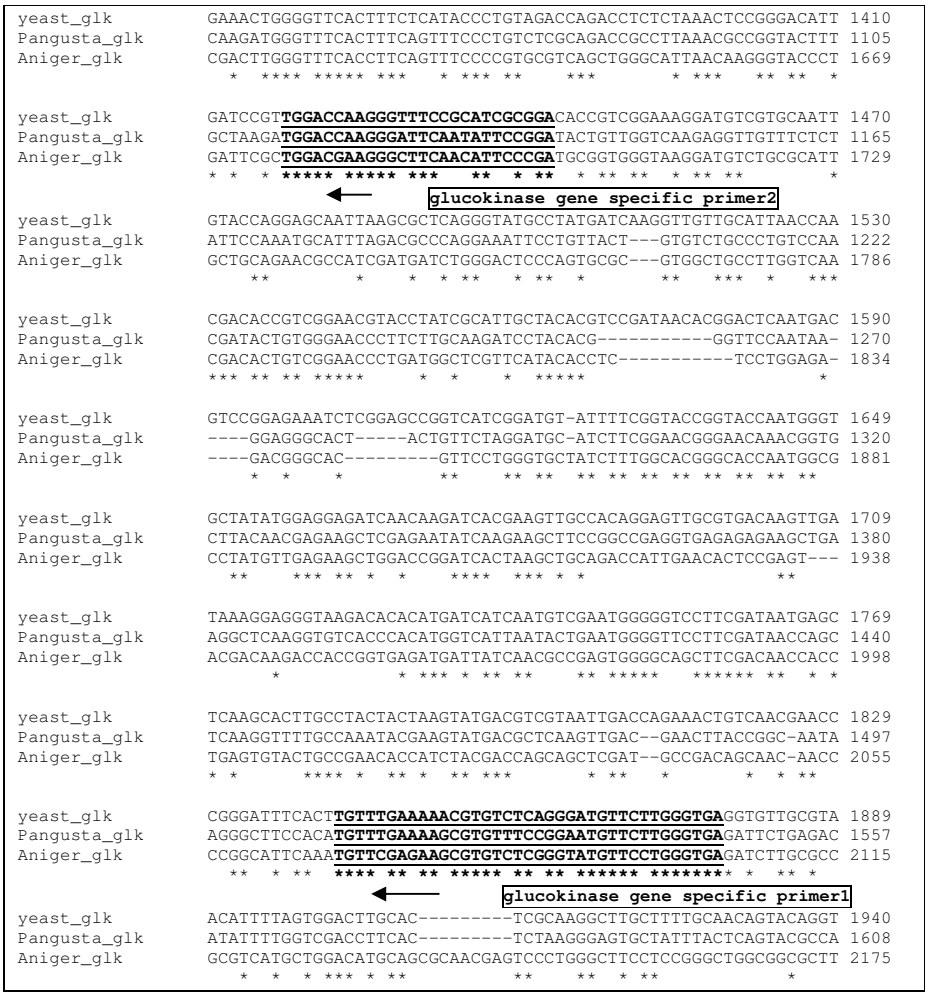

Figure 3.16: The ClustalW analysis of the gene sequences used for designing gene specific primer 1 and gene specific primer 2 for glucokinase. The sequence labels in the figure stand for: yeast_glk; *S. cerevisiae* glucokinase, Pangusta_glk; *P. angusta* glucokinase, Aniger_glk; *A. niger* glucokinase. The figure shows only a part of the alignment where the primer sequences are highlighted by underlining and bold letters. The directions of the arrows are from 5' to 3' end of the primers and the names of the primers are given in the boxes above the regions that the primers are based on.

In order to test the PCR conditions given by the manufacturer primary PCR was carried out by using AP1 and a primer based on the 2396-2414 bp region of *R. oryzae* pyruvate decarboxylase gene (*pdcB*) sequence. Using four different libraries

as templates four reactions were carried out with this primer set. There were products with all four reactions and the sizes of the products were all as expected sizes. The expected sizes of the products were calculated from the restriction digestion analysis of the *pdcB* of *R. oryzae* (Figure 3.17).

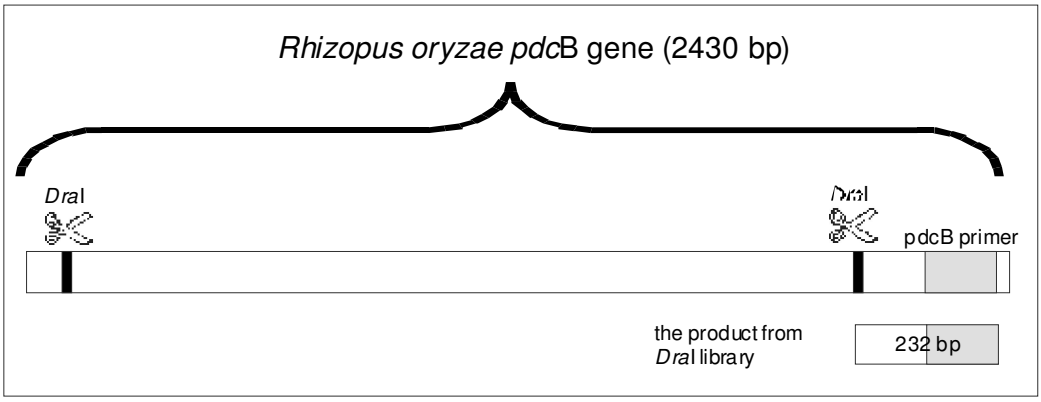

Figure 3.17: The scheme showing the PCR products that could be obtained by GenomeWalker libraries.

As shown schematically in the figure the restriction enzymes *Dra*I cuts twice the *R. oryzae pdc*B. Depending on the distance between the restriction enzyme cut site and the pdcB primer the expected size of the PCR product from GenomeWalker libraries was calculated as 232 bp.

The PCR conditions given by the manufacturer were further optimised since no PCR products with GSP1 for hexokinase and glucokinase were obtained at the given conditions. The annealing and extension temperature of the first 7 cycles was lowered from 72 ˚C to 70 ˚C as well as three different annealing and extension temperatures were tried for the rest of the cycles; 60 ˚C, 62.5 ˚C and 65˚C in a gradient-generating thermocycler (Whatman Biometra TGradient). There were products formed in all the reactions carried out at different temperatures with hexokinase gene specific primer 1 (Figure 3.18), but there were no products in any of the reactions, which were performed with glucokinase gene specific primer. For generating a part of the glucokinase gene the primer designed for nested PCR was used for the primary PCR as well. However, there was no PCR product at any extension temperature tested.

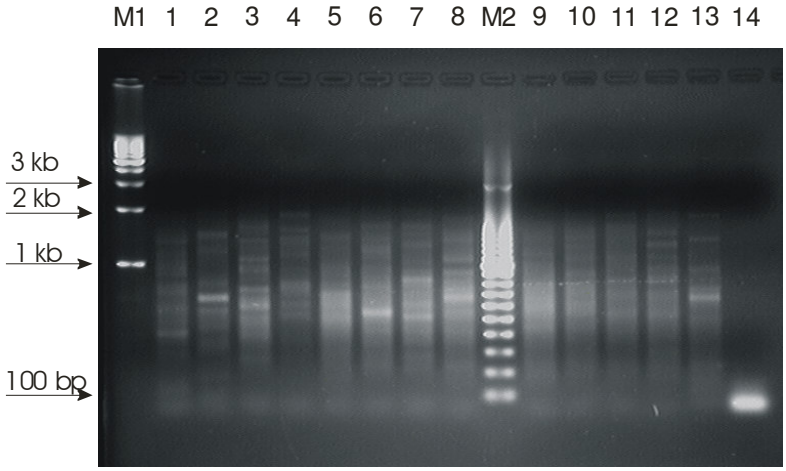

Figure 3.18: The primary PCR products obtained at different annealing and extension temperatures; Lane 1-4 at 60 ºC, Lane 5-8 at 62.5 ºC, Lane 9-12 at 65 ºC. Groups of four are composed of products from four different libraries *Dra*I, *Pvu*II, *Stu* I and *Eco*RV, respectively. Lane marked as M1 contains 1 kb ruler (Biorad) and M2 contains 100 bp ruler (Biorad). Lane 13 is the positive control supplied by the kit. Lane 14 contains a positive control with *Pvu* II library as the template and the *pdcB* end primer and AP2 primer as the primer set.

The diluted primary PCR samples for hexokinase were then used as a template for nested PCR. Figure 3.19 shows the products obtained after nested PCR.

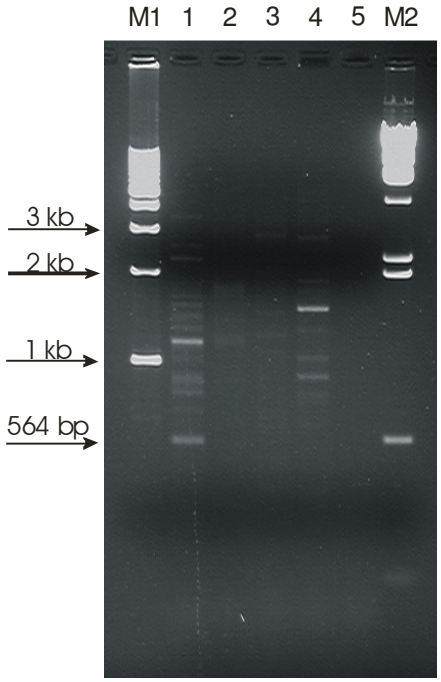

Figure 3.19: The secondary PCR products. Lane 1-4 contains the products of the four libraries : *Dra* I, *Pvu* II, *Stu* I and *Eco* RV respectively. The lane marked as M1 contains 1-kb ruler as a marker (Biorad) and the lane marked as M2 contains *Hin*D3 digested λ DNA marker.

3.4.2. Cloning and sequencing PCR products

A total of 45 white and pale blue colonies were collected from the transformation plates, the plasmids obtained from these colonies were cut with *Eco*RI to check if they contain inserts. Fourteen of the clones were found to contain inserts with varying sizes. The sizes of the inserts were between 300 bp and 3500 bp. Ten of these clones were sequenced by GATC-reaction mix (Amersham Pharmacia Biotech) using SP6 and T7 primers and their characteristics are displayed in Table 3.6.

Table 3.6: The sizes of the inserts contained in the clones obtained from transformation of *E. coli* DH5α and the sizes of the sequences obtained by sequencing using SP6 and/or T7 primer.

Name of the clone	Size of the insert (bp)
DL1-4	1300
DL1-5	1900
DL1-9	500
DL1-10	2300
DL2-8	1200
DL2-10	2000
DL3-2	550
DL4-5	1500
DL4-8	1800
DL4-14	550

3.4.3. Analysis of the sequences from libraries

3.4.3.1. Analysis of the sequences by blast tool

The analysis of the sequence by tblastx and blastx search tool did not give any hits to hexokinase or glucokinase sequences from any other organisms. There were some hits to some hypothetical proteins from some filamentous fungi e.g., *Neurospora crassa*, some sequences which look like ATP-binding LON gene of *S. cerevisiae* (Table 3.7).

Table 3.7: The tblastx and blastx results of the library clones.

The name of the library clone	Name of the protein	Score bits	E value
dl4-14 _T7	*S. cerevisiae* PIM1 gene for mitochondrial ATP-dependent protease	55	$1e^{-18}$
	S. cerevisiae LON mRNA	55	$1e^{-18}$
dl4-14_SP6	*Paracoccidioides brasiliensis* lon proteinase gene	64	$2e^{-13}$
	S. cerevisiae (LON) mRNA	62	$7e^{-10}$
	Neurospora crassa hypothetical protein	60	$1e^{-08}$
	S. cerevisiae mitochondrial ATP-dependent protease:Pim1p	57	$9e^{-08}$
	Chylamydophila pneumoniae CWL029 Lon ATP-dependent protease	41	$5e^{-07}$
dl1-10_sp6	*Branchiostoma belcheri* ribosomal protein S25	75	$6e^{-13}$
	S. cerevisiae ribosomal protein S25.e.B, cytosolic	75	$6e^{-13}$
	Spodoptera frugiperda ribosomal protein S25	71	$8e^{-12}$

As was mentioned in the introduction part (section 1.1.1.1.) the genome sequence of *R. oryzae* will be available within months. The un-assembled clone sequences of *R. oryzae* genome project became available. In order to find putative hexokinases and glucokinases in *R. oryzae* a bioinformatic approach was conducted, where aminoacid sequences of closely related fungal species were blasted against the *R. oryzae* genome. The organisms and protein sequences used for search are hexokinase protein (Hxk) sequences from *Aspergillus oryzae*, *Aspergillus niger*, *Yarrowia lipolytica*, *Kluyveromyces lactis*, *Debaromyces occidentalis*, *Saccharomyces cerevisiae* (both Hxk 1 & Hxk2), *Schizosaccharomyces pombe* (Hxk 2) and glucokinase protein (Glk) sequences from *S. cerevisiae* and *A. niger*. A total of 147 nucleotide sequences, whose translation products were significantly similar to hexokinase and/or glucokinase sequences were aligned using SeqMan program and resulted in 15 contigs. Each contig was then used to make a blast search on NCBI database using the blastx tool to search for their similarities to the known hexokinase and glucokinase protein sequences. All of the contig sequences showed similarities to hexokinase and glucokinase protein sequences from different organisms. These data were then used to determine the start codon, stop codon and intron splicing sites.

Having determined the probable hexokinase and glucokinase nucleotide and protein sequences it was possible to look for the similarity between these sequences and the sequences of the products obtained by the GenomeWalker kit. Each probable gene sequence was aligned with each sequence from the libraries in pairs. The results showed that none of the sequences obtained by GenomeWalker were similar to the probable *hxk* or *glk* sequences. Moreover, the gene specific primer 1 and gene specific primer 2 for hexokinase were searched within the probable gene sequences. Sequences similar to the gene specific primer 1 could not be found in any of the contigs. However, there were some contig sequences that showed similarity to the gene specific primer 2. These contigs containing similarities are contig 2, contig 9 contig 19 and contig 18. Finally the putative hexokinase gene and protein sequences from *R. oryzae* and the gene and protein sequences of glucokinase (Glk) and hexokinase (Hxk) protein sequences from different fungi were aligned by ClustalW tool of MegAlign. The putative protein sequences from *R. oryzae* were aligned as a

separate cluster apart from the glucokinase and hexokinase sequences from other fungi (Figure 3.20).

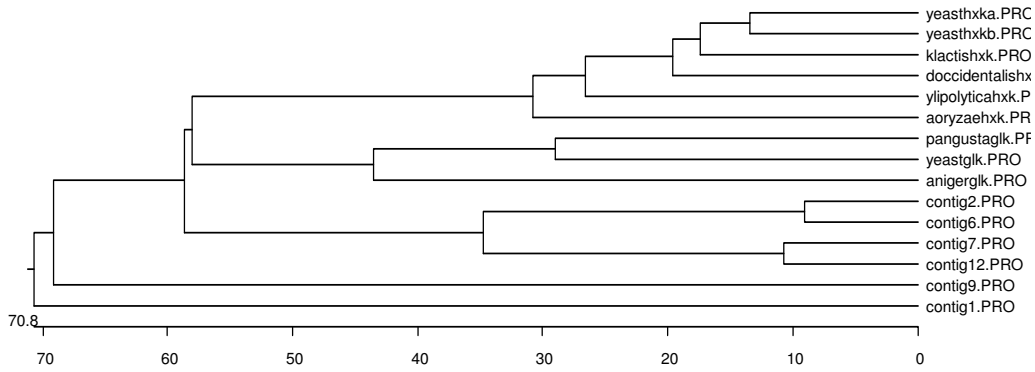

Figure 3.20: ClustalW analysis of the probable hexokinase and/or glucokinase protein sequences from *R. oryzae* and glucokinase and hexokinase sequences from other fungi. The sequences named as contig 1, 2 *etc* represent the sequences from *R. oryzae*. The abbreviations given as names of the sequences from up to the bottom stand for: yeast (*S. cerevisiae*) HxkA, yeast (*S. cerevisiae*) HxkB, *K. lactis* Hxk, *D. occidentalis* Hxk, *Y. lipolytica* Hxk, *A. oryzae* Hxk, *P. angusta* Hxk, yeast (*S. cerevisiae*) Glk, *A. niger* Glk.

3.4.3.2. Southern blot analysis

The results of the southern analysis demonstrated that there were sequences similar to *A. niger* hexokinase sequence in the primary PCR products but there was not any signal in the nested PCR products (Figure 3.21).

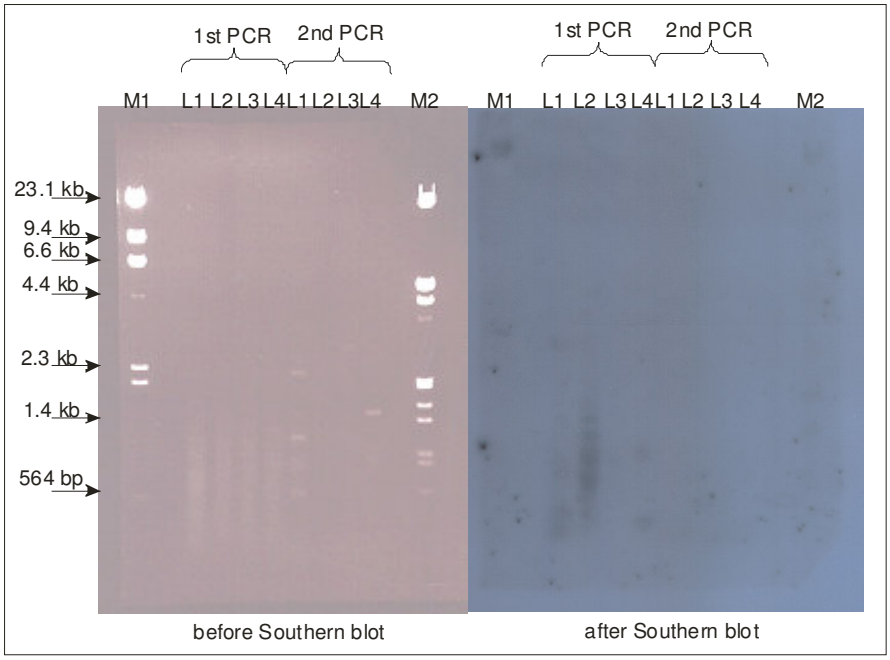

Figure 3.21: Southern blot analysis of primary and nested PCR products.

There was no signal in any of the hybridisation analysis of *Eco*RI-digested *R. oryzae* genomic DNA done using either *A. niger hxk* or *A. niger glkA* as a probe.

CHAPTER 4

DISCUSSION

4.1. Purification and characterisation of pyruvate decarboxylase from *Rhizopus oryzae*

4.1.1. Purification of pyruvate decarboxylase

This study represents the purification of pyruvate decarboxylase (PDC) from *R. oryzae* for the first time. Although some minor bands could be detected, the PDC activity was associated with the major band detected after the silver staining of the gel (Figure 3.4). Moreover, in the SDS PAGE analysis the major band corresponds to a band, which has a molecular weight similar to the expected size of the PDC proteins from *R. oryzae*. This suggests that the major protein in the purified fraction is PDC. Since the activity staining procedure is dependent on the detection of the product of PDC reaction acetaldehyde by non-enzymatic condensation with dianilinoethane [98] this result supported that the major band corresponds to the native PDC of *R. oryzae*. The enzyme was not further purified to homogeneity and the partially purified enzyme was used for further characterisation experiments.

The PDC has two isoenzymes as Skory (2003) cloned and characterised two genes (*pdc*A and *pdc*B) coding two different pyruvate decarboxylase enzymes. The deduced translation products of these two genes have been reported to have 91 % amino acid identity, which was calculated by Lipman-Pearson comparisons. Both of the genes are expressed under different conditions [16]. The isolelectric points (pI) for these two proteins were calculated by using the GeneRunner program and was found to be 5.36 for PDCA and 5.44 for PDCB. Although the calculations are theoretical, they show that these two enzymes are very similar to each other in terms of their charge. Regarding this, in one of the purification trials we could obtain

elution of activity in two peaks, which are hardly separated (Figure 4.1) in anion exchange chromatography column.

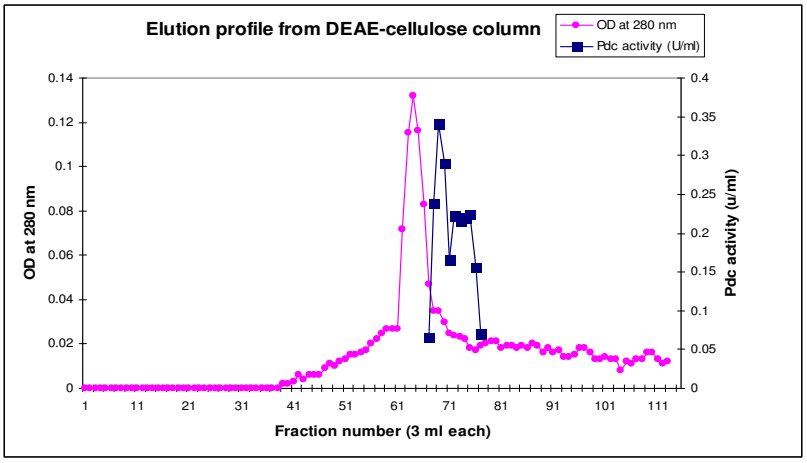

Figure 4.1: The elution profile of DEAE-cellulose column showing a double activity peak of pyruvate decarboxylase. The –●-shows the protein concentration as the absorbance at 280 nm, and -■- represents the PDC activity.

This result was not reproducible which could be caused by the differential expression of the two genes in different batches of cultivation of mycelium used in purification. However, in all the purification trials we could see the tailing of the activity peak, which could be due to having two isoenzymes that are slightly different in their pI values. These facts suggest that the purified fraction could be a mixture of pyruvate decarboxylase isoenzymes. However, using the classical chromatographic methods it would be very difficult to separate these very similar isoenzymes. On the other hand, this study gave some clues about the pyruvate decarboxylase(s) of *R. oryzae* and enlightened our future work on these enzymes and the genes coding them.

4.1.2. Characterisation of pyruvate decarboxylase

The monomer molecular weight of the enzyme found by SDS PAGE analysis (59±2 kDa) corresponds to the value calculated from the amino-acid sequences deduced from the nucleotide sequences of both PDCA (61351 Da) and PDCB (61525

Da) of *R. oryzae* [16]. These values are in agreement with the previously reported subunit-molecular weight of the Pdc's isolated from *S. cerevisiae* (61 kDa) , *K.lactis* (61 kDa) [103],from wheat germ (*Triticum aestivum*) (61-62 kDa and 63-65 kDa) [104], *Oryza sativa* L. (62 and 64 kDa) [105], *Zymomonas mobilis* (59 kDa) [61], *Sarcina ventriculi* (57 ±3 kDa) [106].

Pyruvate decarboxylase from *R. oryzae* shows a sigmoid dependence of the catalytic activity on the substrate concentration (figure 3.6) like the other PDC species from plant seeds and yeasts. Having a slope of 1.8 as the slope of the linear portion of the Hill Plot (Hill coefficient: n_H) implies that the purified PDC from *R. oryzae* behaves as if it possesses at least two sites for substrate binding. These sites are acting in a co-operative manner as n_H is higher than 1. Hill coefficients of other PDC's from other organisms can be listed as 1.9 for *T. aestivum* PDC [104],1.7-1.8 for *S. cerevisiae* [107] all of which have values similar to what we obtained for *R. oryzae* PDC.

The enzyme is inhibited at pyruvate concentrations higher than 30 mM. Inhibition at high pyruvate concentrations has been reported for other PDC species as well. To illustrate, PDC from *K. lactis* is inhibited at a concentration of 100 mM pyruvate with a K_i of 1.2 mM. The K_i values that are reported for other PDC species are much lower than of *K. lactis* being 0.58 M for *Z. mobilis* PDC [62] and 0.2-0.3 M for brewer's yeast (*S. cerevisiae*) PDC [103]. The low level of K_i shows that pyruvate inhibition is much stronger in these organisms compared to *K. lactis* PDC. Altough the inhibitory concentrations of pyruvate for PDC of *R. oryzae* is very high and physiologically irrelevant it shows that there could be a regulatory site for binding of the pyruvate other than active site(s). This in turn could be an important characteristic that should be investigated at various conditions, which will mimic particular physiological states *in vivo*.

The optimum pH was estimated to be around 6.5. This value is not very specific since the pH dependence of the enzyme was found to be in a broad range. But the estimated value for optimum pH is in line with the values found for pyruvate decarboxylases from other organisms. The optimum pH's of several different organisms are reported as 6.0 for *K. lactis* [103], 6.8 for yeast, 6.0 and 6.25 for *Z. mobilis* [60, 62], 6.3-6.7 for *S. ventriculi* [106], 6.25 for *O. sativa* [105].

One of the regulatory factors, which affect the partitioning between lactate and ethanol branches of the pyruvate branch point could be the pH. Depending on the physiological state and the intracellular pH the production of ethanol or lactate could be favoured in *R. oryzae*. In fact this was suggested for the ethanol and lactate production metabolism of plants [53, 108-111]. According to the widely accepted Davies-Roberts pH-stat hypothesis, the plants primary response to oxygen limitation is a burst in lactate production, which causes a drop in the intracellular pH. This leads to the activation of PDC and inhibition of LDH [108] thereby causing a shift from lactic acid to ethanolic fermentation. This hypothesis is based on the fact that the pH's in which the LDH and PDC enzymes are active is alkaline for Ldh and acidic for PDC [108]. Although the condition of lactate production is different for *R. oryzae* this hypothesis can hold for its metabolism as well. As it was reported earlier that LDH from *R. oryzae* has an optimum pH of 7.5 [27] and we have found the optimum pH for PDC of *R. oryzae* as 6.5 (this study). The changes in pH could be regulating the channelling of the pyruvate via regulating the activities of these two branch point enzymes. In this respect the intracellular pH value of the *R. oryzae* and its regulation is an essential information which should be known before concluding anymore about the regulatory role of pH on the pyruvate branch point.

Other than their optimum pH, another factor, which could be affecting the partitioning between lactate and ethanol, could be the difference in their affinities for their common substrate. The K_m of *R. oryzae* LDH for pyruvate is 0.64 mM [27] while that of *R. oryzae* PDC is 8.6 mM (this study), which makes the LDH a more likely candidate to be active when the pyruvate concentration levels are low.

For comparison of the characteristics of PDC species from various organisms a table showing all the discussed parameters above is given in Table 4.1.

Table 4.1: Comparison of the characteristics of pyruvate decarboxylases from different organisms. The abbreviations stand for: *Saccharomeyces carlsbergensis*, *Zymomonas mobilis*, *Kluyveromyces lactis*, *Sarcina ventriculi*, *Zea mays*, *Pisum sativum* and *Rhizopus oryzae* respectively.

The name of the organism	Monomer molecular weight (kDa)	pH optimum	Km (mM)	Hill coefficient	Inhibition by high pyruvate concnetration	references
S.carlsbergensis	60	6.0	0.47	-	Yes with a K_i of 0.2-0.3	[103]
Z. mobilis	59±1	6.5	0.3	1.4	no	[60]
K. lactis	61.5	6.0	0.3	-	Yes with a K_i of 1.2	[103]
S. ventriculi	57±3	6.3-6.7	13	-	-	[106]
Z. mays	58-60	5.8	4.5-7.6	3.2-1.4	no	[57]
P.sativum	65-68	6.0	1	-	-	[112]
R. oryzae	59±2	6.5	8.6	1.8	Above 30 mM	This study

The sudden drop in the enzyme activity at alkaline conditions can be attributed to the loss of metal ions (Mg^{2+}) at alkaline pH's [61, 62]. In both Pdc enzymes from *S. cerevisiae* and *Z. mobilis* the cofactors (Mg^{2+} and TPP) dissociate from holoenzyme at pH values above 8.0 and recombine tightly with apoenzyme at pH 6.5 to yield the active enzyme as reported by Diefenbach *et al* (1991).

Although *R. oryzae* is not primarily producing ethanol it has been reported to be able to convert 38 % of the glucose into ethanol under the given conditions [113]. The strain used in this study is NRRL 395 and is the same strain with the one we used in our study so we can use it as a reference for our study. As can be calculated from the purification yield and the purification fold the pyruvate decarboxylase protein makes up the 6-9 % of the soluble cell protein. The reported value for the % of pyruvate decarboxylase in the soluble cell protein for *Z. mobilis* PDC is between 4-6 % of the soluble protein [62]. Although the % of PDC in the soluble protein fraction for *R. oryzae* and *Z. mobilis* are comparable the ethanol production rates are quite different being 95 % for *Z. mobilis* and 38 % for *R. oryzae*. Taking this into account and trying to find what is the difference between these two organisms one can make a list of differences of which one of them is the drastic difference in the affinity of the two pyruvate decarboxylases towards pyruvate. The K_m of *R. oryzae*

PDC is 8.6 mM whereas, K_m of *Z. mobilis* PDC is 0.4 mM. Another very important difference is that *Z. mobilis* PDC obeys the normal Michaelis-Menten kinetics while *R. oryzae* PDC shows a sigmoidal dependence to its substrate, pyruvate.

4.2. PCR cloning of lactate dehydrogenase A (*ldh*A), lactate dehydrogenase B (*ldh*B), pyruvate decarboxylase A (*pdc*A) and pyruvate decarboxylase B (*pdc*B)of *Rhizopus oryz*ae and transformation of *Aspergillus niger* with these genes

4.2.1. PCR cloning of lactate dehydrogenase A (*ldh*A), lactate dehydrogenase B (*ldh*B), pyruvate decarboxylase A (*pdc*A) and pyruvate decarboxylase B (*pdc*B)of *Rhizopus oryz*ae

This part of the study involved the cloning of *ldh*A, *ldh*B, *pdc*A, and *pdc*B from *Rhizopus oryzae*. Cloning of the genes coding the pyruvate branch point enzymes were achieved by PCR cloning since these genes were already cloned [16, 25]. The aim is to study the regulation of the pyruvate branch point in *R. oryzae*. This part of the study was successful in that we cloned all four genes. From the sequence of the genes obtained it is clear they do not contain any mutations that could alter the translation product of these genes.

4.2.2. Transformation of *Aspergillus niger* NW219 with *ldh*A, *ldh*B, *pdc*A and *pdc*B of *Rhizopus oryzae*

Transformation of *A. niger* NW219 was done with *ldh*A, *ldh*B, *pdc*A and *pdc*B of *R. oryzae*. The co-transformation frequencies for the transformations done with *ldh*A and *ldh*B of *R. oryzae* were of the expected value being in the range of 40-60 %. These values were very low for the transformations done with *pdc*A and *pdc*B of *R. oryzae* being lower than 20 %. These differences in the transformation frequency could be due the high copy number integration of the plasmid containing the *pdc*A and *pdc*B genes resulting in high expression and as a result of that a

reduced growth of the transformants. The growth rate of the *pdc*1(pyruvate decarboxylase 1)-overproducing strain of *Saccharomyces cerevisiae* was shown to have 10 % lower specific growth rate compared to the wild type strain [12]. Pyruvate decarboxylase (PDC) reaction uses pyruvate as substrate as well as the pyruvate dehydrogenase complex (PDH) does. Channelling of pyruvate into fermentative branch in excess amounts could lead to impairment of growth. Growth of the organism, biomass production, needs the action of TCA (tricarboxylic acid cycle), in which the energy equivalents (NADH, FADH2) are produced (Figure 1.3). Pyruvate dehydrogenase complex is the first step of this cycle and it shares pyruvate with the Pdc enzyme. It was shown that induction of *R. oryzae pdc* genes resulted in enhanced ethanol production [16]. Thus we can conclude that overexpression of *pdc* could result in increased formation of ethanol by increasing the substrate supply for the subsequent reaction, alcohol dehydrogenase, which consumes NADH. This will further cause a decrease in the NADH that is needed for growth. Apart from the effect of specific activity or regulation of the introduced gene, even the production of a metabolically inactive protein was shown to reduce the specific growth rate due to dilution of the other enzyme activities [114].

Regarding the lactate dehydrogenase transformants we do not expect a decrease in their growth rate, since the lactate branch is inhibited by lactic acid [115], as can be observed in Figure 3.3. The control of the lactate branch with the product of its reaction may not allow the continuous action of this branch.

As was presented in Table 3.1 and Table 3.2 some of the transformants containing the gene of interest (*ldh*A, *ldh*B, *pdc*A or *pdc*B) showed to have lower specific activities of the corresponding enzymes compared to wild type. The data were taken at a single time point and not all the variables like residual glucose in the medium and the biomass produced were known. This suggests that there may be differences at their growth phases i.e. some could be at the mid-exponential phase while the others are at their late exponential phase. The integration of the plasmid carrying the gene is random and could have been into any part of the genome which would affect the physiology of the strain. Moreover, the enzyme activity measurements were done in the crude extract which contains various amounts of

enzyme activities which might have interfered with the lactate dehydrogenase or pyruvate decarboxylase enzyme activity measurements.

Higher citrate production of all of the *ldh*A and *ldh*B transformants compared to wild type can not be explained with the available data but we can suggest some possibilities which might have an influence on this. One of the prerequisite for a high rate of acid formation is an undisturbed metabolic flow through glycolysis [116]. Moreover, it was shown that increased rate of glycolysis could lead to increased rate of citric acid production [2, 47, 48, 117]. First of all overproduction of lactate dehydrogenase gene (*ldh*A or *ldh*B of *R. oryzae*) could have introduced the change in the pH of the cytosol or the timing of pH change into *A. niger* NW219. This could have an influence on the activities of the glycolytic enzymes or other regulatory reactions. Secondly increase in the production of cytosolic NADH could have been changed. Finally the transport of lactate out of the cell could have altered the intracellular ion concentrations (Na^+ or K^+) depending on the type of transport used for transport of lactate. This in turn can affect the enzyme activities that are using these ions as cofactors, e.g. K^+ is used as cofactor by pyruvate kinase which is one of the enzymes in the glycolysis.

No gluconate production of the *ldh*A and *ldh*B transformants could be due to rapid use of glucose in the metabolism. Gluconic acid production is a process taking place out of the cell and consists of simple dehydrogenation of glucose by the action of glucose oxidase [118]. Under normal circumstances *A. niger* produces gluconic acid during the exponential phase and it is parallel to cell growth [119, 120]. Lactate dehydrogenase transformants having an increased rate of lactate production thus might have been utilising the glucose at an increased rate could have led to no production of gluconate under the conditions tested.

As the data of transformation studies suggest further analyses of all the transformants is necessary. To determine clearly whether the transformants contain any of the genes from *R. oryzae*, Southern blot analysis should be done using the whole gene(s) as homologous probes. Furthermore, northern blot analysis is necessary to show that the genes are expressed. The set of data including mycelium sample for biomass determination, enzyme activity measurements, DNA isolation, RNA isolation and medium samples to determine the organic acid production and

residual glucose concentration should be taken simultaneously. This will eliminate differences between the strains which could be predicted and which will arise experimentally.

4.4. Genomic library construction

The attempts to make a genomic DNA library from *R. oryzae* were not successful, as it was not possible to obtain fragments migrating along with the current in agarose gel electrophoresis. The same procedure was applied several times. However, the fragments obtained after each recovery showed the same pattern.

Replacing agarose with LMP-agarose was not successful either. The reason was that it was not possible to obtain agarose-free sample of DNA. Moreover, the DNA was not detectable by agarose gel electrophoresis if there was any at all.

The methods used for the genomic library construction from *R. oryzae* were the procedures which were used for *A. niger* in the same laboratory (Wageningen University, Fungal Genomics). The reason of not being successful can be attributed to the differences between these organisms. For example if you compare the GC content of the DNA sequences from two organisms the GC contents were drastically different in that A. niger has a GC content of ~50 % while that of R. oryzae is ~40%. The GC content can be calculated in EditSeq program by using any of the gene sequences from *A. niger* or *R. oryzae*. Moreover the low GC content of several *Rhizopus* species was studied and it was found out that the GC content of these organisms ranged from 35% to 40 % [121]. Moreover our personal experience showed that the DNA isolation protocols used routinely for *A. niger* (Appendix K) was not suitable for *R. oryzae* thus we optimised another protocol which is given in section 2.2.2.1.1. for DNA isolation from *R. oryzae*. Thus a genomic library construction from R. oryzae can be achieved by use of different protocols.

4.5. Cloning genes from *Rhizopus oryzae*

The evaluation of the technique suggested that it could be used as a substitute for screening genomic library. The critical point is the design of the primers. The control reactions with *pdc*B primer gave the expected sizes of products suggesting that the products obtained are most probably the fragments of *pdcB*. Whereas, the products obtained by degenerate primers (GSP1 and GSP2) of hexokinase didn't show any similarity to hexokinase sequences of other fungi and of probable hexokinase and/or glucokinase sequences *of R. oryzae*.

Analysis of the probable Hxk and Glk sequences showed that they are not quite similar to the HXK's and GLK's of the other fungi (Figure 3.20). This finding also can explain why there were not any PCR products when GSP1 and GSP2 for *glk* were used. Moreover, the degenerate primer sequences for hxk were not very homologous to the probable nucleotide sequences from *R.oryzae*.

The Southern blot analyses of *Eco*RI-digested genomic DNA of *R. oryzae* with *hxk* and *glkA* of *A. niger* (Figure 3.21) suggest that the similarities between probable gene sequences of hexokinase and/or glucokinase of *R. oryzae* and the *hxk* and *glk*A sequences of *A. niger* are very low. In order to be able to obtain a signal in heterologous hybridisation the amount of DNA used for Southern blot analysis should be high. The presence of signal in the Southern blot analysis of primary PCR products support this idea. Apparently there are some sequences which are homologous to *hxk* of *A. niger* in the primary PCR products and the amount of these fragments are relatively high on the gel. There might be sequences which are homologous to *hxk* of *A. niger* in nested PCR products as well. However, since the total amount of the DNA on the gel was lower for nested PCR compared to the amount loaded for primary PCR there was no signal obtained.

Finally it can be stated that the method was not successful in obtaining a fragment or fragments of *hxk* and/or *glk*. The method is more likely to be successful when the primers designed from known sequences are used, in this case for *pdc*B primer. Since the *R. oryzae* genome database is available, primers which are more

homologous to *R. oryzae* gene sequences can be designed. This will in turn allow us to use this method for cloning genes from *R. oryzae*.

4.6. Conclusion

The current work on *Rhizopus oryzae* allowed us to have some clues about the regulation at pyruvate branch point in this organism. Some of the kinetic characteristics revealed by the purification and characterisation of pyruvate decarboxylase from this fungus gave a better view about the regulation at pyruvate branch point as well as it brought some more questions to answer. We have seen that there are two isozymes which can not be separated by the methods we have used and the question brought by this finding is "Why this organism needs two very similar enzymes for the 'same' function?". For further studies their kinetic characteristics should be analysed by purifying them separately. The regulation of the expression of these two isoenzymes at various physiological conditions is another point that should be investigated and by using the cloned genes as homologous probes for the northern blot analysis.

The second part of the study involved cloning of lactate dehydrogenase and pyruvate decarboxylase genes from *Rhizopus oryzae* and their expression in *A. niger*. This part was helpful in that these enzymes which are not purified separately until now can be purified from *A. niger* transformed with *ldh*A, *ldh*B, *pdc*A or *pdc*B of *R. oryzae* since the native enzymes of *A. niger* is expected not to be closely related with enzymes. Supporting this fact the last part of the study in which the cloning of hexokinase and glucokinase genes of *R. oryzae* was the main aim showed that the probable gene sequences obtained from *R. oryzae* genome sequence are not quite similar to the gene sequences from different fungi. The genome sequence of *R. oryzae* which is publicly available will make it easier to design specific primers for cloning genes from this fungus.

All the above mentioned works are necessary to reveal the metabolic regulation of this fungus. Another very essential work that should be done to be able to make strain improvement in *R. oryzae* is to develop a transformation system for this organism. A transformation system will enable us to make overexpression and

deletion mutants of the corresponding genes which is important for strain improvement and it will serve as a tool to study the importance of the enzyme(s) encoded by the mutated gene(s).

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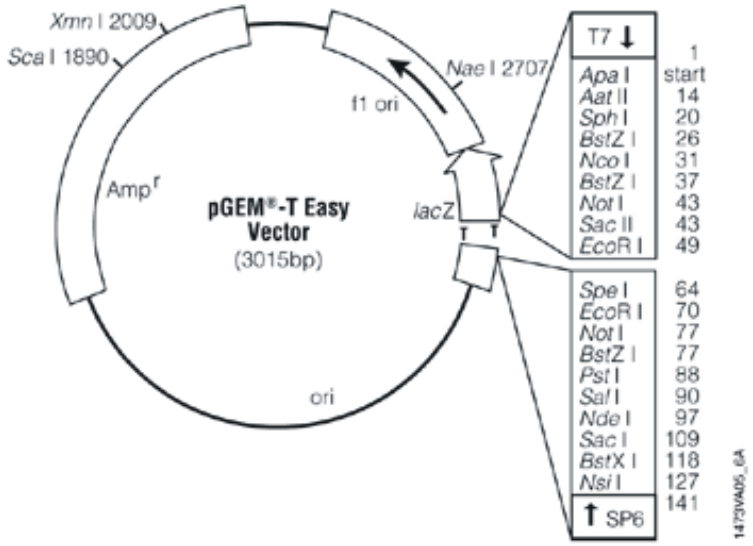
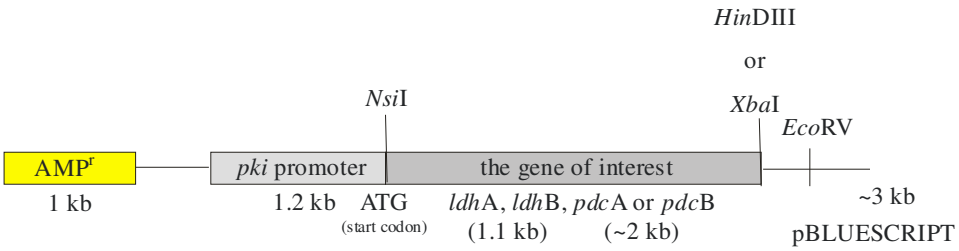
APPENDICES

Appendix A: Plasmid maps

The PGEM T-easy vector map (taken from the technical bulletins given in www.promega.com)



The sketch of the expression vector used for transformation of *Aspergillus niger*



- the terminator sequences are native sequences from the gene fragment
- *pki* promoter is the promoter of pyruvate kinase gene of *Aspergillus niger* and is a strong promoter
- the vector is pBLUESCRIPT and contain ampicilline resistance gene (AMP^r)
- some of the restriction sites are given as abbreviations (*Eco*RV, *Nsi*I, *Xba*I, *Hin*DIII)

Appendix B: Growth media of *Escherichia coli* DH5α

Luria Bertani (LB) medium composition :

For 1 L	
Yeast extract	5 gr
Tryptone	10 gr
NaCl	10 gr
Agar	15 gr

Adjust the pH of the medium to 7.0 by addition of 5 N NaOH (~0.2 ml).

The stock concentration for ampicilline is 50 mg/ml in dH_2O. The final concentration used in the medium is 20 µg/ml.

Appendix C: Growth media for *Rhizopus oryzae*

Salt medium composition (defined media)

Chemicals	**Grams/100 ml**	**Final concentration**
Ammonium sulfate $(NH_4)_2SO_4$	0.2	15 mM
Potassium phosphate (KH_2PO_4)	0.065	5 mM
Magnesium sulfate ($MgSO_4.7H_2O$)	0.025	1 mM
Zinc sulfate ($ZnSO_4.7H_2O$)	0.005	0.2 µM
Glucose (anhydrous)	0.5	28 mM
Agar	2	2 % (w/v)

The above composition is used for solid media but it can also be used as liquid defined media by exclusion of agar and addition of variable concentration of glucose.

Rich medium composition (complex media)

Chemical	**Grams/100 ml**	**Final concentration**
NaCl	0.5	86 mM
Peptone	1	1 % (w/v)
Yeast extract	0.5	0.5 % (w/v)
Glucose	2	111 mM

Appendix D: Media for *Aspergillus niger*

Minimal Medium

Minimal medium salts for 1000 ml:

6.0 g $NaNO_3$

1.5 g KH_2PO_4

0.5 g KCl

0.5 g $MgSO_4 . 7\ H_2O$

1 ml Vishniac trace-elements solution

pH adjusted to 6.0 before sterilisation. For solid medium 15 g agar is added.

50 mM carbon source and appropriate supplements are added after sterilisation

Complete Medium for 1000 ml:

Minimal medium salts +

1 ml Vishniac trace elements solution

2 g meat peptone (pepton 100)

1 g yeast extract

1 g pepton 140 or Casamino acids (vitamins free)

0.3 g yeast ribonucleic acids.

2 ml vitamins solution

pH adjusted to 6.0 before sterilisation. For solid medium 15 g agar is added

1 % carbon source and appropriate supplements (1.5 mM leucine, 8 μM nicotinamide and 5 mM uridine) are added after sterilisation.

Vishniac solution (Vishniac and Santer, 1957):

10 g EDTA

4.4 g $ZnSO_4.7H_2O$

1.0 g $MnCl_2.4H_2O$

0.32 g $CoCl_2.6H_2O$

0.32 g $CuSO_4.5H_2O$

0.22 g $(NH_4)_6Mo_7O_{24}.4H_2O$

1.47 g $CaCl_2.2H_2O$

1.0 g $FeSO_4.7H_2O$

pH is adjusted to 4.0 and stored at 4 ºC.

Vitamins solution

10 mg thiamine

100 mg ribovlavine-5P

10 mg p-aminobezoic acid

100 mg nicotinamide

50 mg pyridoxine-HCl

10 mg panthothenic acid

2 mg biotine

are dissolved in 100 ml demineralized water

Supplement stock solutions:

marker	supplement	mg/100 ml	dilution in media
nic	nicotinamide	20	1/200
pyr	uridine	12200	1/100
leu	leucine	2000	1/100

Uridine should be sterilised by passing it through 0.2 µm filter, leucine and nicotinamide stock solutions can be sterilised by autoclaving.

Appendix E: Silver staining of the polyacrylamide gels

Reagents:

A) Fixer:

150 ml methanol + 36 ml acetic acid + 150 µl of 37 % formaldehyde and complete to 300 ml with dH_2O

B) 50 % ethanol : prepare 1 L of 50 % ethanol.

C) Pretreatment solution (Sodium thiosulfate solution):

Dissolve 0.08 g sodium thiosulfate ($Na_2S_2O_3.5H_2O$) in 400 ml dH_2O, mix with a glass rod and take 8 ml aside for further use in preparation of developing solution.

D) Silver nitrate solution:

Dissolve 0.8 g silver nitrate in 400 ml dH_2O and add 300 µl 37 % formaldehyde

E) Developing solution:

In an Erlenmeyer flask measure 9 g potassium carbonate and add 8 ml previously kept pretreatment solution and 300 µl 37 % formaldehyde. Complete the volume to 400 ml with dH_2O

F) Stop solution:

In an Erlenmeyer flask mix 200 ml methanol and 48 ml acetic acid and complete to 400 ml with dH_2O.

Procedure:

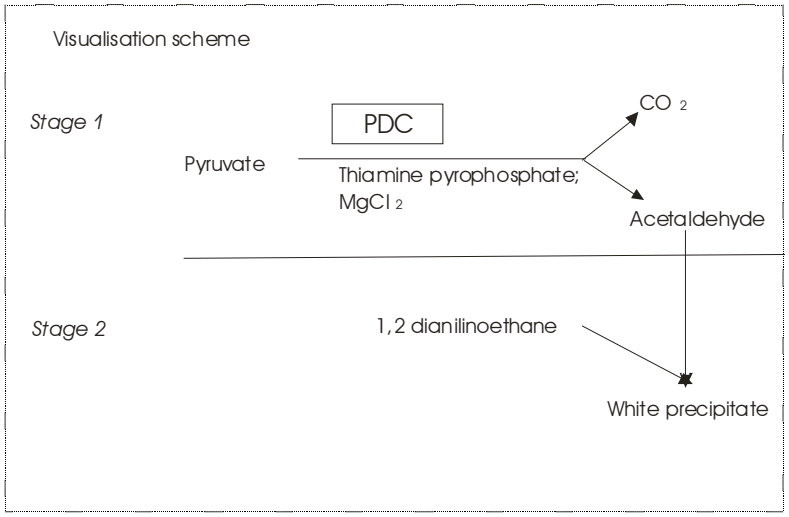
The procedure given in the below table are followed. All the steps were performed by constant shaking on aplatform shaker.

	Step	**Solution**	**Time**	**Comments**
1	Fixing	Fixer	>1 hour	
2	Washing	50 % EtOH	3 X 20 min	
3	Pretreatment	Pretreatment solution	1 min	Time should be exact
4	Rinse	dH_2O	3 X 20 sec	Time should be exact
5	Impregnate	Silver nitrate solution	20 min	
6	Rinse	dH_2O	2 X 20 sec	Time should be exact
7	Developing	Developing solution	~5 min *	
8	Wash	dH_2O	2 X 2 min	
9	Stop	Stop solution	>10 min	

- Time is adjusted by ones self according to the color development.

Appendix F: Activity staining of pyruvate decarboxylase

The principle underlying the acitivity staining of pyruvate decarboxylase is given in the figure as a scheme below.



Staining solution:

A. 0.3 M Sodium citrate buffer, pH 6.0

30 mM Sodium pyruvate

5 mM Mg SO4

5 mM Thiamin pyrophosphate

B. 100 mg 1,2 dianilinoethane dissolved in 10 ml glacial acetic acid and total volume adjusted to 35 ml with water.

Procedure:

Incubate the electrophorised gel in 200 ml of solution A at room temperature for 50 minutes and then add 3 ml solution B. Opaque bands clearly visible as white precipitates on the semi-transparent gel appear after o/n incubation. Wash the stained gel with water and photograph.

Appendix G: Solutions for DNA isolation from *Rhizopus oryzae*

TE Buffer (10 mM Tris HCl pH 8.0 and 1 mM EDTA)

10 % sodium dodecyl sulfate (SDS)

20 mg/ml proteinase K (stored in small single use aliquots at -20 ºC)

5 M NaCl

CTAB/NaCl solution (10 % CTAB in 0.7 M NaCl)

Chloroform / isoamyl alcohol (24:1) (The ratio is given as volume/volume)

Phenol/chloroform/isoamyl alcohol (25: 24:1) (The ratio is given as volume/volume)

Isopropanol

70 % ethanol (diluted with dH_2O)

Appendix H: Transformation of *Escherichia coli* DH5α

Materials:

Luria Bertani Medium (LB): Dissolve 10 gram bacto-tryptone, 5 gram bacto-yeast extract and 10 gram NaCl in 950 ml distilled water. Adjust the pH to 7.0 with 5 N NaOH and complete the volume to 1 litre. Sterilise by autoclaving

X-gal: 2 % solution in dimethyl formamide (20 mg in 1 ml). Final concentration of X-gal in the LB plates and broth should be 0.04 mg/ml.

Ampicilline: 1 % solution in distilled water (10 mg in 1 ml) Sterilise the solution by filtering it through sterile 0.2 µm filters. Final concentration of ampicilline in the LB plates and broth should be 0.01 mg/ml.

Frozen competent cells are stored in -70 ºC freezer. Each tube contains 250 µl of competent cells. You will usually use 50 µl of competent cells per transformation treatment. When removing competent cells from the freezer do not leave the door open longer than necessary. Take an ice bucket to the freezer. Take a box containing competent cells out of the freezer and close the door. Remove the required number of tubes to the ice bucket and replace the box immediately in the freezer being sure to hook the door shut.

1) Thaw competent cells on ice (approximately 20 minutes)

2) While cells are thawing, set up microfuge tubes with DNA for your transformation treatments. You will also need a tube containing no DNA for a transformation for a negative control. Keep the tubes on ice. (DNA volume can be maximum 1/10 th of the cell volume)

3) When cells are thawed, make sure the cells are resuspended (pipette up and down gently with a yellow tip)and transfer aliquots of 50 µl to each treatment tube including the negative control. Be sure not to cross conyaminate the tubes. Use a fresh yellow tip for each transfer if necessary.

4) Incubate the DNA + cells on ice for 30 minutes.

5) Heat shock the cells by placing the tubes at 42 ºC for 2 minutes

6) Add 0.5 ml of LB without antibiotics to each treatment without cross contaminating the treatments.

7) Incubate the tubes at 37 ºC for 1 hour

8) Plate 200 µl of the treatment on LB plates + antibiotics (+ X-gal if required). Alternatively, all the cells can be plated by spinning the tube for 1 minute, pouring off the supernatant and resuspending the cells in the remaining supernatant prior to plating. The supernatant should be poured into a glass container and autoclaved.

9) Incubate the plates overnight at 37ºC. The plates may then be sealed and stored at 4 ºC for about 1 month (for most strains).

Appendix I: Plasmid DNA isolation (fast Mini-prep)

Materials:

Buffer S1: 50 mM Tris/HCl pH 8.0
10 mM EDTA
400 ug/ml DNase-free RNase A

RNase A solution is made DNase-free by boiling for 5 min and is added after sterilisation of buffer S1. The RNase containing buffer is stored at 4^{o}C.

Buffer S2: 200 mM NaOH 1 % SDS
Freshly prepared by mixing equal volumes of 0.4 M NaOH 2 % SDS storage at room temperature

Buffer S3: K-acetate buffer pH 5.2: Mix 60 ml 5 M K-acetate
11.5 ml acetic acid
28.5 ml water

Protocol:

1 Inoculate 5 ml LB medium (10 ul from glycerol stock or a single colony from a plate) and incubate overnight at 37^{o}C.

2 Pipette 2 ml in eppendorf tube and centrifuge for 5 min at 5000 rpm.

3 Remove supernatant and centrifuge for 1 min and carefully remove the rest of the supernatant.

4 Resuspend the pellet in 100 ul buffer S1 (5 min on a plate vortex)

5 Add 100 ul buffer S2 and mix manually.

6 Add 100 ul buffer S3, mix manually and leave on ice for 5 min.

7 Centrifuge for 15 min at 14000 rpm at 4^{o}C.

8 Transfer the supernatant to a 1.5 ml Eppendorf tube and add 200 ul isopropanol, mix manually and centrifuge for 15 min at 14000 rpm at 4^{o}C.

9 Remove the supernatant, add 0.5 ml 70% ethanol to the pellet and centrifuge for 5 min at 14000 rpm at 4^{o}C.

10 Remove the supernatant and briefly dry the pellet under vacuum.

11 dissolve the pellet in 20 ul sterile water or TE.

Appendix J: Preparation of spore suspension and spore count from *Aspergillus niger*

Materials:

Sterile Pipettetes and Pipetteting tips

Sterile saline-Tween solution (0.9 % NaCl + 0.005 % (v/v) Tween-80

Protocol:

1. Pipette 10 ml saline-Tween solution on the spore-mat.

2. Scrape off the spores using a bend inoculation needle.

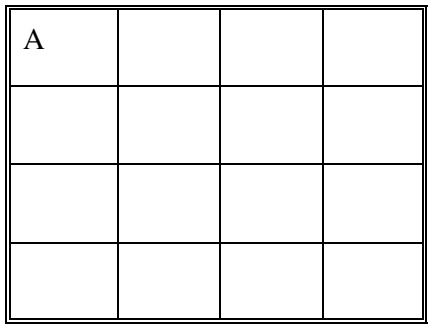
3. Transfer the spores suspension to a sterile bottle with a sterile plastic pasteur pipette.

4. Prepare a 20-fold dilution (50 ul + 950 ul saline-Tween) and determine the concentration of spores by counting the diluted sample in a heamocytometer (see remarks).

5. Calculate the concentration of spores as described below

Neubauer improved haemocytometer is used for counting spores. The depth of the area is 0.1 mm and the minimal area shown as with 'A' has an area of 1/400 mm^2

(magnification 400 x)



The spores are counted (magnification 400 X) in 16 fields.

The concentration of spores in the spores suspension is calculated using the formula below.

spores/ml = count x d x 2.5 x 10^5 (d = dilution)

Appendix K: DNA isolation from *Aspergillus niger*

Reference: L.H. de Graaff (1989), The structure and expression of the pyruvatekinase gene of *Aspergillus nidulans* and *Aspergillus niger*, PhD thesis Agricultural University Wageningen.(Revised for use of 2 ml Eppendorf tubes)

Materials:

Büchner flask and funnel
Dnase-free Eppendorf tubes
micro dismembrator with shake flask and grinding balls
liquid nitrogen container, pair of forceps, safety spectacles
and micro centrifuge

Recipes:

TE buffer:
10 mM Tris/HCl pH 7.6
1 mM EDTA

5 x RNB per 1000 ml:
121.10 g Tris/HCl pH 8.5
73.04 g NaCl
95.10 g EGTA

DNA extraction buffer:
1.0 ml tri-isopropyl-naphtalene (0.02 g/ml in water)
1.0 ml p-amino-salicylic acid (0.12 g/ml in water)
0.5 ml 5 x RNB

phenol: (Equilibrated with Tris/HCl buffer pH 8.0 c.f. Maniatis)

chloroform

phenol/chloroform/isoamylalcohol (25 :24 :1)

isopropanol

70 % ethanol

Protocol:

1. Inoculate 250 ml culture medium with 1 * 10^6 spores/ml and grow overnight at 30 ^oC and 250 rpm in a shake incubator
2. Harvest mycelium on a piece of nylon gauze in a Büchner funnel, using mild suction, wash two times with approximately 100 ml of saline.
3. Dry the mycelial mat by pressing it between several layers of bench paper, cut into pieces and freeze in plastic vial, in liquid nitrogen. Mycelium can be stored at - 70 ^oC at this point
4. Prepare DNA extraction buffer.
5. Precool shake flask, grinding balls, forceps and spatula in liquid nitrogen.
6. Weigh approximately 0.2 g of frozen mycelium and submerge in liquid nitrogen in shake flask together with the grinding balls.
7. Poor off excess liquid nitrogen, cap shake flask and quickly install the assembly in the dismembrator.
8. Grind mycelium for 1 minutes at approximately 2/3 of the max. amplitude.
9. Take the shake flask assembly out of the dismembrator (Carefully, pressure build up inside the flask may cause the cap to fly off), and open carefully (see above).
10. Transfer the grinded mycelium to a 2 ml Eppendorf tube using a precooled spatula and immediately add 0.9 ml of DNA extraction buffer and 0.5 ml phenol and vortex.
11. Leave the tube for 5 min at room temperature.
12. Add 0.4 ml ml of chloroform and vortex.
13. Centrifugate for 10 min in Eppendorf centrifuge at max. speed
14. Transfer the aqeous phase (clear upper layer, discard the interface and bottom layer) to a new 1.5 ml Eppendorf tube.
15. Add 0.5 ml phenol/chloroform (1 : 1) and vortex.
16. Centrifugate for 10 min in Eppendorf centrifuge at max. speed and transfer upper layer to a new 1.5 ml Eppendorf tube.

17. Add 0.5 ml chloroform and vortex

18. Centrifugate for 10 min at max speed and transfer upper layer to a new 1.5 ml Eppendorf tube.

19.Repeat this step until the interphase is absent.

20. Add 0.8 vol. isopropanol and mix gently. Centrifuge for 10 min at max. speed and discard the supernatant..

21. Wash the pellet with 0.5 ml 70 % ethanol.

22. Centrifugate for 10 min at max speed and discard the supernatant.

23. Air dry the pellet.

24. Dissolve the DNA in 200 µl TE with 50 µg/ml DNAse-free RNAse and incubate at 37 ºC for 30 min.

25. Determine the concentration of the DNA by agarose gel electrophoresis: Prepare 2, 5 and 10 fold dilutions in TE and run out on an agarose gel next to 50, 100, and 200 ng of lambda DNA. Alternative method: Pipet 4 µl DNA solution into 800 µl water and measure the absorbance at 260 and 280 nm. OD260 = 1 equals [DNA] =10 µg/µl. For high quality DNA OD260/280 > 1.8.

Appendix L: Southern blot analysis

Capillary blotting of DNA

1) Make a picture of the gel with a ruler next to the gel. Incubate the gel for 30 min in 0.25 M HCl.

2) Incubate the gel, with shaking, 30 minutes in denaturation buffer (1 M NaOH)

3)Incubate the gel, with shaking, 30 minutes in neutralisation buffer (1 M Tris/HCl pH 7.4, 1.5 M NaCl)

4) During incubation steps: cut a piece of nylon membrane slightly larger than the gel, cut 3 pieces of Whatman paper at the same size as the nylon membrane. Dilute 1 L of 20 x SSC to obtain 2 L of 10 x SSC. Prepare the "blotting table"

5) Put the gel on the "blotting table"; remove all air bubbles using a test tube

6) Use pieces of plastic foil to prevent buffer to pass around the gel

7) Wet the nylon membrane with 2 x SSC and put it on top of the gel; remove all air bubbles

8) Wet the pieces of Whatman paper and put them on top of the nylon membrane

9) Put a stack of paper towels on top of the Whatman paper and a 1 kg weight.

10) Leave overnight or over the weekend

11) Remove the paper towels and the Whatman paper.

12) Mark the position of the slots through the gel using a pencil, wash the membrane in 5 x SSC.

13) Remove the gel and air dry the membrane for 30 minutes.

14) Fix the DNA to the membrane by cross-linking on a UV transilluminator for 2 min.

Prehybridisation and hybridisation

Materials:

Hybridisation- oven, tubes and gauze, fragmented salmon sperm DNA (ss DNA, 5 mg/ml in sterile bidistilled water).

Recipes:

Hybridisation buffer: 6 x SSC, 5 x Denhardt's, 0.01 M EDTA, 0.5 % SDS

20 x SSC: 175.3g NaCl , 88.2 g Sodiumcitrate and make up to 1 l with bidistilled water

100 x Denhardts: 10 g ficoll, 10 g polyvinylpyrolidone, 10 g BSA make up to 500 ml, store in aliquots at - 20 ^{o}C

2 x SSC wash buffer: 100 ml of 20 x SSC, 50 ml of 10 % SDS and make up to 1 L with bidistilled water

0.2 x SSC wash buffer: 10 ml of 20 x SSC, 50 ml of 10 % SDS and make up to 1 L with bidistilled water

Protocol:

1) Warm hybridisation oven and buffer at 68 ^{o}C, and denature fragmented ssDNA by boiling for 10 minutes (store on ice until further use).

2) Carefully place the blot on a piece of hybridisation gauze and wet with a few ml of 2 x SSC.

3) Carefully remove any air bubbles by gentle rubbing with a wet glove, roll up, and put the roll in a hybridisation tube.

4) Add 10 - 20 ml (approximately 0.1 ml per cm^2 membrane) of prewarmed hybridisation buffer and 250 - 500 ul of the heat denatured ssDNA (10 mg/ml).

5) Close the tube, making sure that the rubber ring is properly installed in the lid, and unroll the membrane by gently rolling the tube in your hands, avoiding air bubbles between the tube and the blot

6) Insert the tube in the oven rotor, making sure that it turns in the same direction as you used to unroll the membrane. Balance the rotor by inserting a dummy tube opposite of your hybridisation tube.

7) After 15 - 30 minutes the tube is checked for leakage. If any leakage is found, disassemble the lid and rubber ring, carefully clean both and reinstall the rubber ring. If there is still leakage try another lid and tube.

8) After 2 hours of prehybridisation, the radiolabelled and heat denatured probe is added to the tube, and the hybridysation is commenced over night. Prewarm wash buffers to be used the next morning at 68 ^{o}C

9) The next morning, the hybridisation mix is carefully poored out of the tube into a 30 ml bottle which, after cooling, is stored at - 20 ^{o}C.

10) Wash the blot by adding 30 - 40 ml of 2 * SSC wash buffer and incubating in the oven at 68 ^{o}C for 20 minutes.

11) Pour of the wash buffer in a liquid waste container and wash once again with 2 x SSC wash buffer, twice with 0.2 x SSC wash buffer, and subsequently rinse a few times with 2 x SSC for 5 minutes (to remove SDS).

12) Unroll the blot by inserting the tube the other way round in the oven during the last rinse, take out the blot, and dry on a piece of Whatman paper.

13) Fix the blot on the Whatman using a few adhesive labels. The position of the blot is indicated with fluorescent labels. Wrap the whole in Saran Wrap before autoradiography.

VITA

Şeyda Açar was born in Afyon on December 31, 1972. She received her B.S. degree in Biology Education from The Middle East Technical University (METU) in June 1997. She received a second B. S. degree in Biology at the same year by completing the double major programme in Biology in METU. She got her M. Sc. Degree in Biology from METUin September 1999. She has been a project assistant in the Graduate School of Natural and Applied Sciences in METU since 1998. During her Ph. D. she has been a guest worker in the Wageningen University, Department of Microbiology, Fungal Genomics group in The Netherlands between July 2002 and December 2003. This study has been supported by Turkish Scientific and Research Council (TÜBİTAK) as a BDP project. Her main areas of interest are fungal physiology and regulation of it.