INVESTIGATION OF SUGAR METABOLISM IN *Rhizopus oryzae*

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ABSTRACT

INVESTIGATION OF SUGAR METABOLISM OF *Rhizopus oryzae*

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Rhizopus oryzae is a filamentous fungus, which can produce high amounts of L(+)-lactic acid and produces ethanol as the main by-product. In an effort to understand the pyruvate branch point of this organism, fermentations under different inoculum and glucose concentrations were carried out.

At low inoculum size ($1x10^3$ spores ml^{-1}), high amount of lactate (78 g l^{-1}) was produced, whereas high ethanol concentration (37 g l^{-1}) was obtained at high inoculum sizes ($1x10^6$ spores ml^{-1}). Decreasing working volume increased lactate production significantly at high inoculum sizes ($1x10^5$ and $1x10^6$ spores ml^{-1}), but did not influenced the physiology at low inoculum sizes ($1x10^3$ and $1x10^4$ spores ml^{-1}).

In shake flask cultures, at low initial glucose concentrations biomass yield was high and lactate and ethanol yields were low. Higher lactate and ethanol and lower biomass yields were obtained by increasing the initial glucose concentrations. In

alginate immobilized, semi-continuous cultures with cell retention, glucose level in the medium was kept at low values. Like in shake flask cultures, as the glucose concentration decreased lactate and ethanol yields decreased and biomass yields increased. Increasing the glucose concentration by a pulse of glucose caused increases in branch point enzyme activities, as well as in concentrations of the metabolites. In fed batch cultures higher biomass yield (0.25 g DCW g glucose^{-1}) could be obtained.

Lactate dehydrogenase was influenced by the inoculum size and glucose concentration more than pyruvate decarboxylase and alcohol dehydrogenase. It showed higher activity at lactate producing fermentations. Unlike lactate dehydrogenase, pyruvate decarboxylase and alcohol dehydrogenase showed high activity even at low glucose concentrations.

Keywords: *Rhizopus oryzae*, pyruvate branch point, L(+)-lactic acid, ethanol

ÖZ

Rhizopus oryzae' NİN ŞEKER METOBOLISMASININ İNCELENMESİ

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Rhizopus oryzae, yüksek miktarda L(+)-laktik asit üretirken, yan ürün olarak da etil alkol üreten filementli bir küftür. Bu organizmanın pirüvat ayrılma noktasını anlamak amacıyla değişik aşı ve glikoz derişimlerinde fermantasyonlar yapıldı.

Düşük aşı derişiminde ($1x10^3$ spor ml^{-1}) yüksek laktat derişimi (78 g l^{-1}) elde edilebilirken yüksek etil alkol derişimleri (37 g l^{-1}) yüksek inoculum derişimlerinde ($1x10^6$ spor ml^{-1}) elde edildi. Yüksek aşı konsantrasyonlarında ($1x10^5$ and $1x10^6$ spor ml^{-1}) çalışma hacminin düşürülmesi laktat üretimini artırdı, ama hacim değişikliği düşük aşı derişimlerde ($1x10^3$ and $1x10^4$ spor ml^{-1}) etki göstermedi.

R. oryzae'nın düşük glikoz derişimlerindeki fizyolojisi çalkalamalı kültür ve fermentörde incelendi. Çalkalamalı kültürde, düşük başlangıç glikoz derişimlerinde biyokütle verimi yüksek, laktat ve etil alkol verimleri düşük oldu. Başlangıç glikoz derişimini yükselterek daha yüksek laktat ve etil alkol derişimleri elde edildi.

Aljinata tutuklanmış yarı kesikli *R. oryzae* kültürlerinde glikoz çok düşük derişimlerde tutuldu. Çalkalamalı kültürdekine benzer şekilde, glikoz derişimi düştükçe laktat ve etil alkol derişimi düşmüş, biyokütle verimi yükseldi. Glikoz derişimi birden artırıldığında, pirüvat ayrılma noktası enzimlerinin faaliyeti ve metabolit derişimlerinde artışa sebep oldu. Yarı-kesikli kültürde, daha Yüksek biyokütle verimi (0.25 g kuru hücre ağırlığı g glikoz^{-1})elde edildi.

Laktat dehidrogenaz, aşı derişiminden ve glikoz konsantrasyonundan pirüvat dekarboksilaz ve alkol dehidrogenaza göre daha fazla etkilenmiştir. Bu enzim laktat üreten kültürde daha yüksek aktivite gösterdi. Laktat dehidrogenazın tersine, pirüvat dekarboksilaz ve alkol dehidrogenaz düşük glikoz konsantrasyonlarında bile yüksek aktivite gösterdi.

Anahtar Kelimeler: *Rhizopus oryzae*, pirüvat ayrılma noktası, L-(+)-laktik asit, etil alkol

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ADH	Alcohol dehydrogenase
C_E	Ethanol concentration (g l^{-1})
C_G	Glucose concentration (g l^{-1})
$C_{G,feed}$	Feed glucose concentration (g l^{-1})
$C_{G,reactor}$	Reactor glucose concentration (g l^{-1})
C_L	Lactate concentration (g l^{-1})
DCW	Dry cell weight (g)
DTE	1,4-Dithioerythritol
EDTA	Ethelyne diamine tetra acetic acid
FUM	Fumarase
GRAS	Generally recognized as safe
LDH	Lactate dehydrogenase
LAB	Lactic acid bacteria
lpm	Liter per minute
MES	2-[N-Morpholino]ethanesulfonic acid
MDH	Malate dehydrogenase
PDC	Pyruvate decarboxylase
PDH	Pyruvate dehydrogenase
PMSF	Phenyl methyl sulfonyl fluoride
S_o	Initial glucose concentration (g l^{-1})
Tris	trishydroxymethylaminomethane
TM	Trypton-Minerals
vvm	Volume per volume per minute
Y_{SE}	Ethanol yield (g ethanol g $glucose^{-1}$)
Y_{SL}	Lactate yield (g lactate g $glucose^{-1}$)
Y_{SX}	Biomass yield (g DCW g $glucose^{-1}$)
Y_{XE}	Yield (g ethanol g DCW^{-1})

Y_{XL}	Yield (g lactate g DCW^{-1})
X	Biomass concentration (g l^{-1})

CHAPTER 1

INTRODUCTION

Rhizopus oryzae is an industrially important filamentous fungus, which is capable of producing high amounts of L(+)-lactic acid. Some strains can produce fumaric acid. Ethanol was produced in fermentations and has generally been considered as by-product. However, with the increase in interest in bioethanol production, ethanol production by *R. oryzae* also draws attention. Investigation of pyruvate branch point of the organisms is of great interest because pyruvate lies in the junction of many pathways; *e.g.* lactic, fumaric and ethanol producing pathways and tricarboxylic acid cycle, which is the most efficient energy yielding pathway. The possible overflow at the pyruvate level should result in production of the partially oxidized organic acids and ethanol, like the ethanol production in yeasts. In the literature, attempts are made, generally to increase product yield by optimizing the nutritional and physical conditions for fermentation. In order to better understand the distribution at the pyruvate level, regulation of the pathways should be elucidated by more in-depth analysis. The targets for genetic engineering can be determined by characterizing the enzymes and metabolites of the pyruvate branch point as well as glycolysis and other central pathways. Following, the properties of the organism and of some of the pyruvate branch point enzymes are presented as well as general ideas about the regulation of sugar metabolism and branch point by referring mainly to the studies on yeast.

1.1. *Rhizopus oryzae*

Rhizopus oryzae is a filamentous fungus, which is an active component in some traditional foods of Indonesia, China and Japan. It is an obligate aerobe and it

lacks septation. Their asexual spores are contained within sporangia borne on sporangiophores (19). Full classification of *R. oryzae* is given as: Eukaryota; Fungi/Metazoa group; Fungi; Zygomycota; Zygomycetes; Mucorales; Mucoraceae; *Rhizopus*. The synonyms of this fungus are: *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*. *R. oryzae* is known to produce lactic acid (41) and fumaric acids (35). There are also studies on the production of ethanol (79), lipase (67), glucoamylase (88), xylanase (4), pectinolytic enzymes (65) and chitosan (24) using this fungus.

1.2. Lactic Acid

1.2.1. Uses of Lactic Acid

Lactic acid and its salts have widely been used in industry. Main applications are in food, chemical, pharmaceutical and cosmetic industries. Worldwide production of lactic acid was 40 000 tons year^{-1} in 1990 (10), 120 000 tons per year in 2006 (9).

Since it is non-volatile, odorless and classified as GRAS, it has wide applications in the food industry, such as flavouring, pH regulation, inhibition of microbial spoilage, mineral fortification, acidulation and pickling agent. Esters of lactate salts are used as emulsifying agents for bakery goods. Lactic acid and ethyl lactate are used in pharmaceutical and cosmetic industries, in topical ointments, lotions, anti acne solutions, humectants, parenteral solutions, in dialysis for anti carries agents and biodegradable polymers for medical applications (10; 49). Lactate is preferred for this applications, since it has water retaining capacity and skin lightening property and it is a natural ingredient of the human body (83). Technical grade lactic acid is used for leather tanning and textile finishing (49). Currently, lactic acid draws a great attention as a feedstock for the poly lactic acid production, which is biodegradable. In medicine, PLA are used as sutures,

orthopedic implants and in controlled drug release (49). It is also a good packaging material for food products, since it is transparent and degrades slowly by hydrolysis (10). Other potential applications of lactic acid and its derivatives are oxygenated chemicals, green chemicals and growth regulators (10; 9).

1.2.2. Industrial Lactic Acid Production

Lactic acid can be produced chemically and biotechnologically. The chemical route produces racemic lactic acid, while fermentative production can produce the desired isomer. Ninety percent of the worldwide production of lactic acid is by fermentative route (27). In chemical route hydrogen cyanide is added to acetaldehyde to yield lactonitrile, which is recovered and distilled. It is hydrolyzed to lactic acid by HCl or by H_2SO_4. Methyl lactate is produce by esterification of lactate with methanol. Methyl lactate is recovered and purified by distillation and hydrolyzed by water under acid catalysis to produce lactic acid.

Commercial fermentative lactic acid production relies on lactic acid bacteria, mainly the genus *Lactobacillus* (10; 83). Carbohydrate feedstocks (molasses, corn syrup, whey, dextrose, sugar) are used as the carbon source and proteinaceous and complex nutrients are supplied in the form of yeast extract, corn steep liquor, soy hydrolysate, etc. Acid formed is neutralized by excess $CaCO_3$. Carbohydrate, which is around 10% is utilized in 4-6 days in batch fermentation (10). Lactic acid yield is around 90% (See Appendix A for definition of yields). Fermentation broth is filtered to remove cells, carbon treated, evaporated. Ca salt of lactic acid in the broth is acidified with sulphuric acid to get lactic acid and resulting insoluble $CaSO_4$ (gypsum) is removed by filtration. Lactic acid is purified by carbon and ion exchange columns and evaporated. Esterification, distillation and hydrolysis can be used for further purification.

Lactic acid yield of bacterial fermentation is high, however LAB have complex nutritional requirements, which increases the raw material cost and adds purification steps. In addition to these, lactic acid isomer is not 100% pure. Producing pure L(+)-lactic acid and having the ability to grow on inorganic

nitrogen salts, causes *R. oryzae* to draw attention. However, lower lactic acid yield than LAB prevents its commercial use.

1.3. Organic Acid and Ethanol Production by *Rhizopus oryzae*

1.3.1. Lactic Acid

Fungal lactic acid production draws attention, since it has simpler nutritional requirements than lactic acid bacteria (LAB), thus product clarification is easier. However, it requires vigorous aeration for lactic acid production (80) and lactic acid yield is lower than LAB, due to producing ethanol and fumaric acid as by-products. Recently, the number of reports on the fungal lactic acid production has increased, since the demand for poly L-lactic acid is increasing (9). *R. oryzae* produces enantiometrically pure L-lactic acid, which is the preferred feedstock for poly-lactic acid production.

The first submerged cultivation of *R. oryzae* for lactic acid production was described in 1936 (41). In this study, Lockwood and Ward (41) found the optimum conditions for lactic acid production by *R. oryzae* including glucose concentration, temperature and concentrations of mineral salts. Their findings about the nutritional requirements for lactic acid production are still being used today. *R. oryzae* converts glucose to lactic acid aerobically, while limited O_2 concentrations in the culture medium increases ethanol production (80). The fungus can also utilize starch directly to produce lactic acid (23; 28). It is also reported to produce lactic acid from xylose (43). In recent years there have been attempts to utilize wastes for lactic acid production, such as potato starch wastewater (28; 29), food processing waste streams (30) waste office paper (53)and corncobs (62; 3; 47). Yin *et. al.* investigated the effect of various carbon sources on lactic aid production by *R. oryzae* and obtained the highest yield by corn starch either liquefied by α-amylase (82%, based on initial carbohydrate concentration) or hydrolyzed by HCl (79%), followed by glucose (77%), fructose (73%), mannose (70%) and sucrose (52%). Yields obtained by galactose and pentoses were found to be not significant

(below 3%). Ammonia nitrogen can be utilized by the fungus; $(NH_4)_2SO_4$ or NH_4NO_3 are sufficient for the lactic acid fermentation (41). Sugar and the nitrogen source were supplemented with K^+, Mg^{+2}, Zn^{+2}, SO_4^- and PO_4^-. $CaCO_3$ is used generally to neutralize the acid formed.

Lactic acid yields have typically been below 85% and productivity is around 2 g/L $\cdot$h (80). These prevent *R. oryzae* to compete with LAB for industrial lactic acid production. Starting from eighties, reports on fungal lactic acid production are increased, mostly concentrating on the optimizing fungal morphology. Approaches include entrapment of cells in polymeric matrix (25; 22; 12; 77; 84; 80; 14), attachment of cells on a support (12; 76; 84) natural immobilization of cells (80) and promotion of pelleted (36; 85; 2) or mycelial growth(36). These approaches resulted in increased production yields and rates. Increased yields and rates can make fungal lactic acid production economically feasible (44).

1.3.2. Fumaric Acid

Some strains of *R. oryzae* are capable of producing high amounts of fumaric acid. Other Mucorales genera that can produce this acid are *Mucor, Cunnighamella,* and *Circinella.* Rhodes *et. al.*. (1959) examined the effects of several physical and nutritional factors on fumaric acid production by *R. arrhizus* (60). In 3 to 8 days 60 to 70 % of the sugar utilized was converted to fumaric acid at 33 ^oC in vigorously shaken submerged culture. Urea and ammonium sulfate were the most suitable nitrogen sources. Zinc, magnesium, phosphorus, iron and corn steep liquor were necessary for high fumaric acid accumulation (60). Zhou *et al.*. (91) showed that an initial pH of 3.05 and optimal Mg^{+2}, Zn^{+2} and Fe^{+2} concentrations (50 ppm, 4 ppm and 100 ppb, respectively) were optimal conditions for the formation of small (diameter <1 mm), spherical and uniformly distributed pellets, which favored high fumaric acid production. Like in lactic acid producing strains, some of the glucose is converted to ethanol in fumaric acid producing strains. Generally $CaCO_3$ is used to neutralize the acid formed in fumaric acid fermentations. However, calcium fumarate has low solubility, which limits the product concentration in the

fermentation broth (90). In addition carbonate is the source of the CO_2 in the formation of oxaloacetate from pyruvate by pyruvate carboxylase (See Section 1.5.4. Pyruvate Branch Point Enzymes). Fumaric acid accumulation by *R. oryzae* occurs by a cytosolic pathway. Pyruvate is converted to fumarate by the combined activities of pyruvate carboxylase, malate dehydrogenase, and fumarase (51; 35).

1.3.3. Ethanol

There is an increasing interest in biomass based ethanol (bio-ethanol) production. The risk of global warming and shortage and environmental risks of petroleum based fuels promote bioethanol usage. Many filamentous fungus can produce ethanol and some posses xylanase, cellulase and amylase (71). This enables them to carry out simultaneous saccharification and fermentation of biomass, such as agricultural wastes, to ethanol. *S. cerevisiae* has been used widely to produce ethanol; however it does not ferment xylose, the main ingredient in agricultural biomass. Skory et al. (1997) screened several Aspergillus and Rhizopus strains for their ability to ferment simple sugars and some complex substrates. and observed that Rhizopus strains could produce more than 31 g ethanol/l under anaerobic stress from 100 g /l glucose (71). Using 50 g/l initial glucose concentrations 25 g/l ethane was produced. Xylose, cellubiose and corn fiber were also fermented with poorer yields. Paperpulp sulfite liquor (79), wood hydrolyzates (46), corn flour (82), and dilute acid pretreated rice straw (33) were tested as a substrate for ethanol production by Rhizopus strains. *R.* oryzae could ferment the hexoses and pentoses in the paperpulp spent sulfite liquor with a ethanol and lactic yields of 0.37 and 0.30 g/g, respectively (79). From dilute acid wood hyrolyzates, *R. oryzae* produces ethanol with a yield of 0.41 g/g substrate (46). Results show that ethanol production capability of Rhizopus from wastes is promising.

1.4. Morphology of Rhizopus

Morphology of filamentous fungi in submerged culture is of great importance, since it influences the physiology of the organism and the reactor performance.

Filamentous fungi exhibit different growth forms in liquid media; such as freely dispersed mycelia, loose hyphal aggregates (clumps), dense aggregates (pellets) of different sizes (17; 93). The morphology influences the rheology of the fermentation media and the heat and mass transfer properties in the reactor. Dispersed mycelia increases the viscosity of the fermentation broth, which needs more power input to achieve sufficient mixing (17). Pelleted growth does not increase the broth viscosity; however, pellets are not uniform aggregates, in which mass transfer limitation may occur. These different macro-morphologies observed in filamentous fungi strongly affect the transport of nutrients and gases into and out of the mycelial aggregates. Therefore, the metabolites produced and their yields depend on the growth form of the organism (17; 93). Factors that influence the morphology includes agitation, pH, medium components, inoculum size, temperature, substrate concentration and aeration (6; 5; 48; 91; 92; 93; 39). Morphology of Rhizopus spp. has been studied by several authors, generally, in an effort to increase the lactic or fumaric acid yields. Like other filamentous fungi, the morphology of Rhizopus spp. ranged from small and large aggregates to filamentous form. Filamentous form anchors on the internal elements of the bioreactors, such as impellers, probes and baffles. Initial pH values between 2.60 to 3.36 were shown to induce formation of small pellets (<1mm), whereas trace metals had also significant effects on growth type in fumaric acid producing *R. oryzae* (91). Inoculum concentration was shown to strongly affect the morphology in *R. oryzae* (85; 2; 39). Small pellets obtained by adjusting the inoculated spore concentration to the preculture increased the lactic acid yield, while filamentous growth and clumps/congealed masses yield lower lactic acid (85; 2). Metal ions negatively affected the pellet formation, while soybean peptone had a positive effect and potato dextrose broth and calcium carbonate were beneficial to *R. oryzae* for growing small and smooth pellets (39). The effect of peptone on growth type and pellet number was also observed by Byrne and Ward (1989), while the glucose concentration affected only the pellet number (5). Increasing agitation speed caused fungal pellets to decrease in size, while increasing aeration caused an increase (2). The morphology of *R. oryzae* could be manipulated by immobilizing the spores in alginate beads (25; 38; 84), on polyurethane foams (12; 78), on loofa

sponge(16), in polyvinylalcohol-cryogel (14). It was also immobilized naturally in a rotating fibrous bed placed around the impeller in a bioreactor (80). Immobilization generally prevents either formation of viscous fermentation broth or adhesion of the mycelia on the reactor elements, which increases the lactic acid yields. Immobilized cells and pellets can be reused in successive fermentations. Another approach was to use mycelial flocs obtained by adding a mineral support and polyethylene oxide, which also enhanced the lactic acid yield significantly (36; 54).

1.5. Sugar Metabolism and Pyruvate Branch Point

1.5.1. Glycolysis

Microorganisms hydrolyze oligosaccharides, transport sugars into the cell, and oxidize them to pyruvate and catabolize pyruvate (Figure 1.1). Sugar phosphates are cleaved to triose phosphates by either Embden-Mayerhof (often referred as glycolysis) pathway, Entner-Doudoroff pathway or hexose-monophosphate pathway. Triose phosphates are oxidized to pyruvate by Embden-Mayerhof pathway, which is common for all organisms (58). Glycolysis is the central metabolic pathway and is present in almost all of the organisms. In glycolysis one mole of glucose is converted to two moles of pyruvate via 10 enzymatic steps. Glucose, the breakdown product of oligosaccharides and storage polysaccharides, and other carbohydrates are oxidized to pyruvate. In the first part of glycolysis (five reactions), in which energy is invested, the sugar is phosphorylated to fructose-1,6-bisphosphate using two ATPs. This molecule is cleaved to yield two moles of glyceraldehyde-3-phosphate, which is dehydrogenated and phosphorylated to yield 1,3-bisphospo-glycerate. This is the first “high energy compound”, the second is phosphoenol-pyruvate. These transfer their phosphates to ADP, yielding ATP, which process is called “substrate level phosphorylation”. Thus, net two molecules of ATP and two molecules of NADH are produced during glycolysis.

Fermentative capacity of organisms is important in industrial applications, such as production of ethanol and organic acids. A classical application is production of ethanol by *S. cerevisiae* in distillers or as fuel alcohol. Faster utilization of sugar is important for the economy of the processes. Therefore, there have been attempts to improve glycolytic flux of this yeast (66; 26; 74). Flux through a pathway is controlled mainly by the amount of enzymes and by allosteric effectors. It was suggested that flux is controlled at "key points", i.e. at the sugar uptake, and at the irreversible steps: hexokinase, phosphofructokinase and pyruvate kinase (66). Each glycolytic enzyme was overexpressed of *S. cerevisiae* including the above ones and also some pairs of them were overexpressed. No increase in ethanol production rates were observed (66). This questioned the hypothesis that suggested a single flux-controlling enzyme. Similarly, overexpression of phosphofructokinase and pyruvate kinase did not increase citric acid production in *Aspergillus niger* (63). Authors observed decreased fructose-2,6-bisphosphate levels, which could decrease the specific activity of phosphofructokinase. The control may be shared among more than one or all of the glycolytic enzymes. In an effort to increase the fermentative capacity of *S. cerevisiae*, Smits et.al. (2000) overexpressed simultaneously the seven enzymes of the lower part of glycolysis in; glyceraldehydes-3-phosphate dehydrogenase, phosphoglycerate mutase, phosphoglycerate kinase, enolase, pyruvate kinase, pyruvate decarboxylase and alcohol dehydrogenase. The recombinant strain exhibited higher enzyme level than the host strain. Upon a glucose pulse to aerobic glucose-limited continuous culture, specific CO_2 production rate was increased in the host and the recombinant strain. The increase was higher in the recombinant strain, which showed an increased fermentative capacity. However, the ethanol yield was the same in both strains. On the other hand Otterdtedt et al. (52) could alter the mode of metabolism of *S. cerevisiae* by changing a single step. They generated a strain in which glucose uptake was dependent on a chimeric hexose transporter mediating a reduced sugar uptake. This strain respired the glucose even at high glucose levels, i.e., it did not exhibit Crabtree effect (see below).

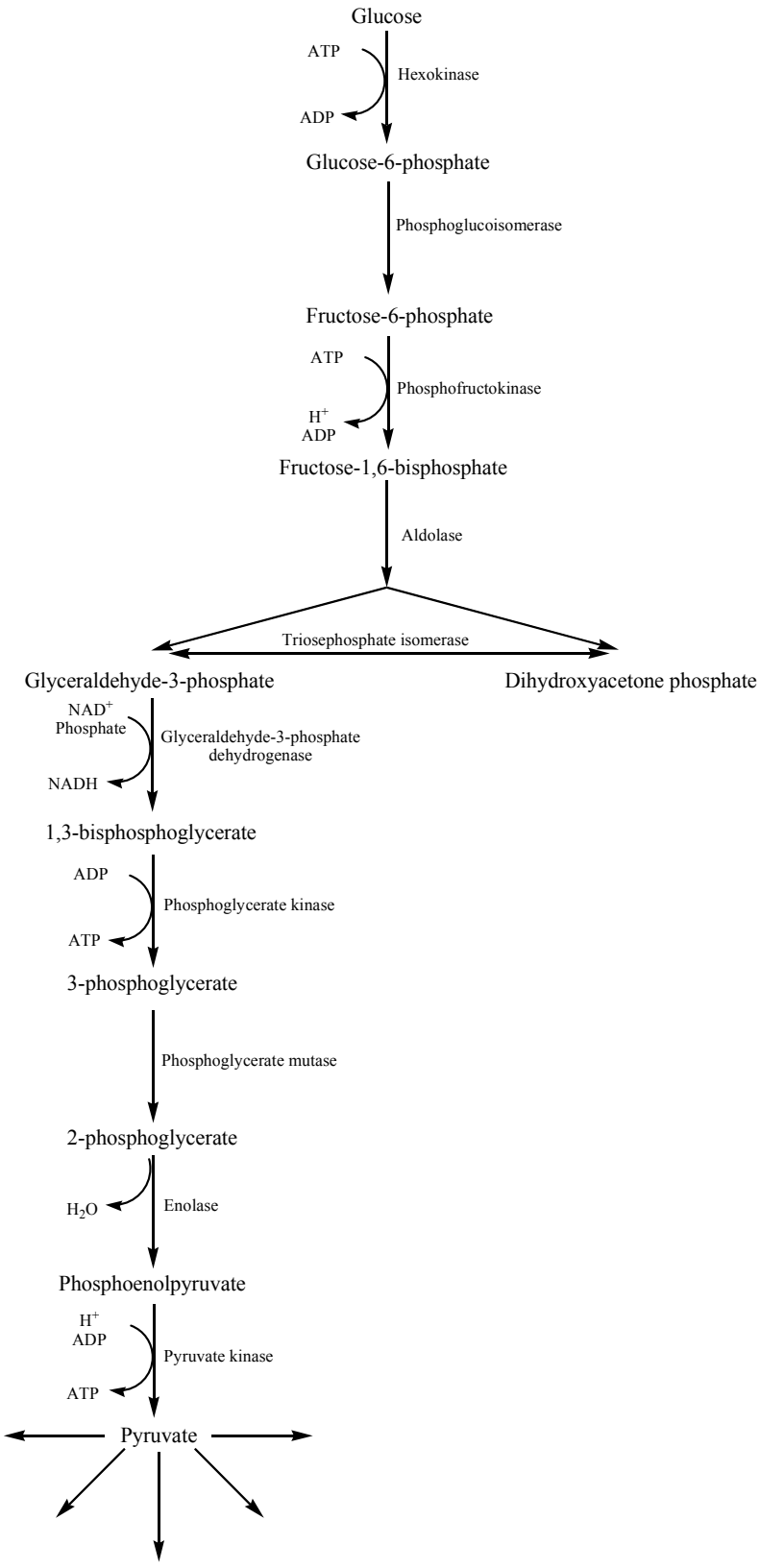


Figure 1.1. Scheme of glycolysis.

1.5.2. Pyruvate metabolism

Pyruvate lies in the junction of many pathways (Figure 1.2). In aerobic organisms pyruvate is oxidized to acetyl-CoA and further oxidized to CO_2 and water in mitochondria via TCA cycle. Reducing equivalents generated in glycolysis and TCA (NADH and FADH) are reoxidized through the electron transport chain. Complete combustion of one mole of glucose in aerobic metabolism yields up to 38 molecules of ATP. In anaerobes pyruvate is not further oxidized. NADH molecules generated in glycolysis are reoxidized to NAD^+ by transferring electrons to an electron acceptor. Some microorganisms can transfer electrons to inorganic ions, while some reduce organic substances. Lactate is one of these organic substances. Pyruvate is reduced to lactate while NADH is reoxidized to NAD^+. Lactic acid bacteria, which are facultative anaerobes, reduce pyruvate mainly to lactate, but acetate, formate, ethanol, acetaldehyde, diacetyl, acetoin and 2,3-butanediol can also be produced from pyruvate (40). Aerobic organisms can also reduce pyruvate rather than further oxidize them under limiting O_2 conditions or pyruvate is produced in excess of mitochondrial capacity. Animal cells reduce pyruvate to lactate upon heavy exercise; when pyruvate is produced faster than that can be metabolized by mitochondria. Yeasts can cleave pyruvate into acetaldehyde and CO_2; acetaldehyde is further reduced to ethanol. This is the generation of alcohol in beverages by yeast fermentation. Baker's yeast uses this mechanism for leavening of dough, where O_2 that is initially present is depleted rapidly leading to anaerobic conditions. In plants, anaerobiosis occurs upon soil flooding and submergence, under which conditions, the organism depends mainly on fermentation as the main energy source. According to Davies-Roberts hypothesis, plants respond to anaerobiosis by producing lactate initially, which causes a drop in cytoplasmic pH. Upon the pH drop, ethanol production is initiated (34).

There are many organic acids produced commercially by biotechnological routes, including citric, gluconic, lactic, itaconic, oxalic, fumaric, malic acids. Their production based on utilization of sugars by specific microorganisms. Ethanol is

another large volume product produced by fermentation by yeast species. Commercial success of biotechnological production of ethanol and organic acid or fungal biomass (such as baker's yeast) depends on rapid (and economical) conversion of sugars to acids and ethanol. Therefore, it is of great importance to characterize the reactions, understanding the mechanisms and regulation of sugar uptake, glycolysis and further oxidation and reduction reactions of pyruvate.

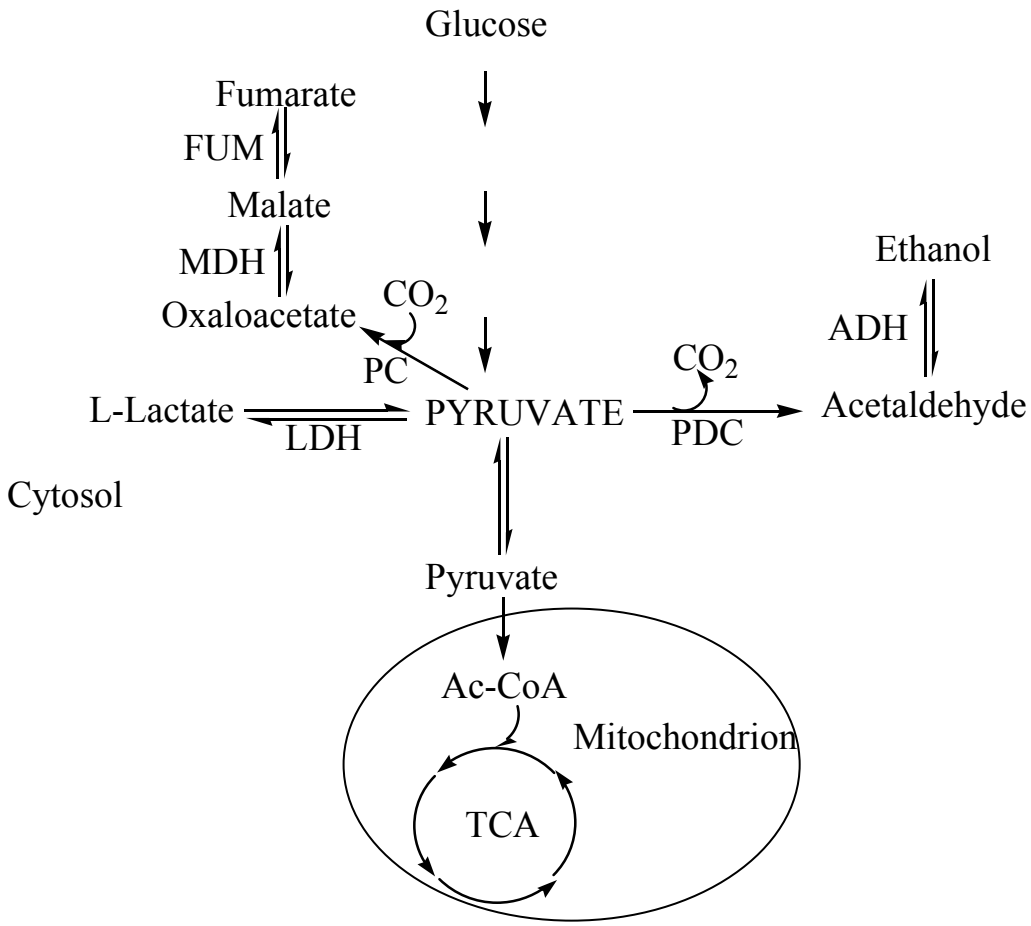


Figure 1.2. Pyruvate branch point of *Rhizopus oryzae*

1.5.3. Regulation of Sugar Metabolism of Yeast

The yeast *S. cerevisiae* has been the subject of many studies, since not only it has been used for centuries for leavening of bread and production of beer and wine, but also it has a distinct sugar metabolism. Sugar metabolism can be considered the most important metabolic activity, thus is the main subject of research. *S. cerevisiae* has been the model organism in cell biology and molecular genetics. The regulation of yeast sugar metabolism may give insights for our studies on *R. oryzae.*

There are four "effects" that regulate sugar metabolism of yeast; Pasteur, Crabtree, Kluywer and Custers effects. They are all related to respiratory and fermentative metabolism. The most prominent one is the Crabtree effect, because it is the main consideration in biomass-directed applications, such as in production of baker's yeast. It is the occurrence of alcoholic fermentation under aerobic conditions (58). At high growth rates, Crabtree positive yeasts, such as *S. cerevisiae*, ferment glucose to ethanol due to low capacity of respiratory pathway for pyruvate dissimilation. When cells growing under sugar limitation are exposed to glucose excess, respiratory route is saturated and excess pyruvate is converted to ethanol. This effect was named after Crabtree, who first showed the repression of respiration in tumor cells (8). De Deken (11) found the same phenomenon in *S. cerevisiae* and concluded that an energy source (respiration) was repressed by another energy source (fermentation). Glucose represses the genes encoding enzymes that are involved in gluconeogenesis, TCA cycle, respiration, mitochondria development and the utilization of carbon sources other than glucose, fructose or mannose (45). Kappeli (32), however, explained the occurrence of respire-fermentative metabolism (fermentation under aerobic conditions) by limited capacity of the respiratory pathway. In glucose limited aerobic continuous culture, O_2 uptake rate increases as dilution rate increases and is constant at the maximum value after the dilution rate at which respirofermentative metabolism starts. This phenomenon is considered in baker's yeast production. In baker's yeast production, cost of the carbohydrate feedstock is

the major factor. Therefore biomass yield on carbohydrate should be high. In fermentative metabolism biomass yield is much lower than in respiratory metabolism. In order to avoid fermentative metabolism, broth is aerated vigorously while sugar is added at the same pace with that of sugar consumption.
Pasteur effect is defined by suppression of alcoholic fermentation in the presence of O_2. It occurs in yeasts that do not exhibit Crabtree effect. In *S. cerevisiae* this can be seen only in slowly growing cells, in which glycolytic flux is low. Custers effect is known as the inhibition of alcoholic fermentation by the absence of oxygen. Yeasts exhibiting this effect can not close the redox balance by producing glycerol or other metabolites under aerobic conditions (58; 61). Some yeasts do not ferment disaccharides, although they can ferment glucose, which is called Kluyver effect. This is due to the control and/or synthesis of the sugar carrier (58). Maximum specific growth rate and biomass yield of *S. cerevisiae* is influenced by the mode of growth. In aerobic batch cultures respirofermentative growth is observed, which is characterized by fast growth but low biomass yield. Full respiratory growth can be achieved in chemostat at low dilution rates and fed-batch cultures, in which sugar is added slowly. The specific glucose uptake rate increases with the specific growth rate up to a point, after which respirofermentative metabolism initiated. Below that critical specific growth rate *S. cerevisiae* produces biomass and CO_2 with a yield on glucose of 0.55 and 0.45 g g^{-1}, respectively. At high glucose uptake rates carbon flow through biomass is lower, around 0.12 g g^{-1}, and rest of the glucose is diverted to ethanol, CO_2 and other products. Under anaerobic conditions; *S. cerevisiae* can grow as long as ergosterol and unsaturated fatty acids are supplied. Energy is generated from glycolysis and ethanol is produced in relation to growth. Biomass yield is around 0.1 g g glucose^{-1}.

1.5.4. Pyruvate Branch Point Enzymes

There are several fates of pyruvate in the cell. In order to increase the flux through the desired branch, the characterization of the pyruvate branch point enzymes and understanding the regulation of the flux distribution are of great importance.

Pyruvate is used as substrate by pyruvate dehydrogenase, pyruvate decarboxylase, pyruvate carboxylase and lactate dehydrogenase. Pyruvate is transported to mitochondria and decarboxylated and oxidized to acetyl-CoA by pyruvate dehydrogenase complex (EC 1.2.4.1). Cytosolic pyruvate can be decarboxylated to acetaldehyde by pyruvate decarboxylase (EC 4.1.1.1), which in turn is reduced to ethanol by the action of alcohol dehydrogenase (EC 1.1.1.1). Another fate of pyruvate in cytosol is the reduction to lactate by lactate dehydrogenase (EC 1.1.1.27). Pyruvate carboxylase (EC 6.4.1.1) catalyzes the ATP dependent carboxylation of pyruvate to oxaloacetate. In yeast, researches generally focused on competition for pyruvate by pyruvate dehydrogenase and pyruvate decarboxylase (58). *R. oryzae* has another competitor for pyruvate, lactate dehydrogenase, which is responsible for high amounts of lactic acid. Fumaric and malic acids are produced via pathway starting by carboxylation of pyruvate by pyruvate decarboxylase in this fungus (44).

1.5.4.1. Lactate Dehydrogenase

Three lactate dehydrogenase have been described in *R. oryzae* (50; 56; 57; 68). The NAD^+-dependent LDH catalyzes reduction of pyruvate by NADH, but not the reverse reaction. It shows high activity at high glucose levels and during the early vegetative growth (57). Enzyme obeyed the Michaelis-Menten kinetics with respect to pyruvate and the K_m values for pyruvate reported were 0.55 (57) and 0.64 mM (89). The K_m for NADH was reported as 0.29 (57) and 0.15 mM (89). Reaction velocity showed a sigmoidal response to NADH concentrations up to 0.03 mM. Lineweaver-Burk plot was linear above this NADH concentration. NAD^+-dependent LDH level decreases when glucose was exhausted in the medium. An NAD^+-independent enzyme replaced and oxidized lactate to pyruvate (56). It can use dichlorophenol indophenol, ferricyanide or cytochrome c but not oxygen as electron acceptors. The K_m for L-lactate was calculated as 3.85 mM (using dichlorophenol indophenol as electron acceptor).

Skory (2000) cloned two genes, *ldhA* and *ldhB*, which encode NAD^+-dependent LDH from *R. oryzae*. LdhA was involved in lactic acid production and was present when glucose, xylose or trehalose was the carbon source. LdhB was present only when the fungus was grown on non fermentable substrates; ethanol, glycerol or lactate. Skory (2004) transformed *R. oryzae* with plasmids containing various lengths of *ldhA* gene fragment and one of the transformants could produce 7% more lactic acid than the parent strain.

1.5.4.2. Pyruvate Decarboxylase

Pyruvate is decarboxylated to acetaldehyde by PDC, which is a TPP and magnesium dependent cytosolic enzyme. Pyruvate decarboxylases of *R. oryzae* were purified and characterized (1). Two isoenzymes had similar characteristics. The Km for pyruvate was found as 3.9 and 4.5 mM for the two isoenzymes (1). Two PDC genes were cloned and they showed 85% nucleotide sequence identity within the coding region (69). In a non fermentable substrate, glycerol, no *pdc* transcript was detected, but induced upon glucose addition. Shifting from aerobic to anaerobic conditions enhanced PDC activity, *pdc* transcript, and ethanol production, compared to aerobic cultures.

In aerobic sugar limited cultures of *S. cerevisiae*, PDC activity increases above the critical dilution rate (growth rate) at which alcoholic fermentation is initiated (55). When three *pdc* genes (*pdc 1, pdc5* and *pdc6*) of *S. cerevisiae* that encodes active PDC were knocked-out, the organism could not grow on glucose as the sole carbon source unless small amounts of ethanol or acetate were added to the medium (15). It was concluded that PDC was indispensable for growth of *S. cerevisiae*, providing the cytosolic acetyl-CoA to be used in lipid synthesis.

1.5.4.3. Alcohol Dehydrogenase

Alcohol dehydrogenase (ADH) reduces acetaldehyde, which is produced from pyruvate by the action of pyruvate decarboxylase, to ethanol by oxidizing NADH.

Two ADH isozymes from *R. javanicus* were characterized (86; 87). Both isozymes have a molecular weight of 62000 and are composed of four subunits. ADH I was thought to be responsible for the ethanol formation, while ADH II was involved in ethanol catabolism. ADH I could oxidize straight chain primary alcohols, whereas ADH II showed broader substrate specificity. NADP was not required as the cofactor for either enzyme (87). The K_m of ADH I for ethanol and NAD were determined as 20.8 mM and 0.311 mM, respectively (86).
S. cerevisiae contains five ADH forms. ADH I is cytosolic and constitutive. It is the major enzyme in fermentative growth, reducing acetaldehyde to ethanol . ADH II is also cytosolic but absent from glucose-grown cells and ADH III is mitochondrial (7). ADH IV is a formaldehyde dehydrogenase and has no effect on ethanol production (13). The function of ADH V is currently unknown (73).

1.5.4.4. Pyruvate Carboxylase

Pyruvate carboxylase is a member of biotin dependent enzyme family and catalyzes the ATP-dependent carboxylation of pyruvate to oxaloacetate (31). Pyruvate carboxylase is localized in cytosol in *R. oryzae* as well as in *S. cerevisiae* and in higher fungi, although in many higher organisms it is a mitochondrial enzyme (51; 58). Malate dehydrogenase and fumarase, which are the other enzymes of the fumarate accumulating pathway are also cytosolic in *R. oryzae* (35). Purified *R. arrhizus* pyruvate carboxylase is partially inhibited by L-aspartate and 2-oxoadipate. It is activated by acyl derivatives of coenzyme (51). In *S. cerevisiae* the K_m values of the enzyme for pyruvate, bicarbonate and ATP were estimated as 0.8 mM, 2.7 mM and 0.24 mM, respectively (58).

1.5.4.5. Pyruvate Dehydrogenase

Pyruvate produced by glycolysis is transported to mitochondria and converted into acetyl-CoA in mitochondria by pyruvate dehydrogenase complex. Complete oxidation of acetyl-CoA by TCA cycle provides high amounts of energy under aerobic conditions. Acetyl-CoA can alternatively be produced by PDH bypass

pathway, by the enzymes pyruvate decarboxylase and acetaldehyde dehydrogenase.

PDH is a large multi enzyme complex. Conversion of pyruvate to acetyl-CoA is catalyzed by the concerted action of the catalytic subunits. In *S. cerevisiae* K_m values of PDH for pyruvate, NAD^+, and CoA are 625 μM, 23 μM and 18 μM, respectively (37). Enzyme shows no cooperativity with respect to pyruvate, unlike the other prokaryotic PDHs. Kinetic and regulatory properties were found similar to the mammalian PDH, except that K_m for pyruvate was much higher than mammalian enzyme, but similar to values reported for the enzyme in Gram negative bacteria (37). Pdh^- *S. cerevisiae* mutants could grow on glucose, indicating that pyruvate dehydrogenase bypass could replace PDH (59). However, the bypass consumes one ATP, therefore biomass yield was lower (0.52 vs. 0.44 g g^{-1} glucose) (59).

1.5.4.6. Competition between Enzymes for Pyruvate

The rate of a metabolic reaction can be controlled *in vivo* by amount (synthesis) of the enzyme catalyzing the reaction, by modification of the enzyme and by the concentrations of the substrates, activators and inhibitors.

Pyruvate concentration may be the main factor that determines the fate of it. K_m for pyruvate of the pyruvate dehydrogenase is an order of magnitude lower than that of pyruvate decarboxylase in *S. cerevisiae.* Therefore, at low intercellular concentrations of pyruvate PDC is by-passed and it is dissimilated by the respiratory pathway. At high concentrations of pyruvate, it is dissimilated by the fermentative pathway producing ethanol. In aerobic chemostat cultures with limiting glucose concentrations, the onset of ethanol production coincided with the increase in the extracellular pyruvate concentration, which was the indicative of the intracellular concentration of it (55). In *S. cerevisiae* the pyruvate branch point enzymes are expressed under all growth conditions investigated, although at different amounts. PDC is present in the cell at high levels irrespective of the O_2

and glucose availability, but it is bypassed in glucose limited respiratory growth. On the other hand, switch to respirofermentative metabolism in *S. cerevisiae* is at least partly due to the limited synthesis of acetaldehyde dehydrogenase, which is a PDH bypass enzyme (58).

1.6. Aim of the Study

The morphology and low lactic acid yield due to ethanol production as by product are the disadvantages of this organism in competing with lactic acid bacteria for industrial lactic acid production. Elucidating the pyruvate branch point can help to increase the lactic acid or ethanol production.

Lactic acid and ethanol portioning at pyruvate branch point of the organism was the main focus of this study. Under some conditions that influenced the lactic acid and ethanol production physiology was defined and supported with activities of pyruvate branch point enzymes. Previous researches in our group and the preliminary work of this study as well as the reports in the literature showed that inoculum concentration influence the physiology and morphology of *R. oryzae*. In the first part of this study we aimed to quantify the effect of inoculum concentration on the physiology of the organism. In the second part, the possible overflow mechanism at the pyruvate branch point was sought.

This study was composed of two main parts: 1) Investigation of the effect of inoculum size and working volume on the physiology of *R. oryzae*, 2) Investigation of the physiology of *R. oryzae* at different glucose concentrations. Both parts included the measurement of the extracellular metabolites, namely, lactate, ethanol, glycerol, as well as biomass and measurement of the activities of lactate dehydrogenase, pyruvate decarboxylase and alcohol dehydrogenase.

CHAPTER 2

MATERIAL AND METHODS

2.1. Chemicals

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

2.2. Organism

Rhizopus oryzae ATCC 9363 (NRRL 395), a lactic acid producing fungal strain, was used in this study.

2.3. Preparation of Spore Suspension

The organism was maintained and sporulated on potato dextrose agar plates at 30 ^{o}C in 4-7 days. Plates were inoculated with 10-20 µl of spore suspension. Fungal mat carrying the spores was separated from agar using forceps and suspended in water. Shaking vigorously spores were transferred from the mat to suspension. Fungal mat was taken out and remaining spore suspension was filtered through a layer of glass wool to remove to carry over mycelia and agar particles. Suspension was centrifuged at 15000 rpm for 5 min (Hettich Microlitre, Germany) and supernatant was discarded. Spore pellet was resuspended in water. Spore concentrations were determined by counting the spores on a haemocytometer. Spore concentration was around $1x10^{7}$-$1x10^{8}$ spores ml^{-1} of the suspension. Spore suspension was used not more than one week.

2.4. Immobilization of Spores in Ca-alginate Beads

In 20-50 ml 2.0% Na-alginate solution $1x10^6$ spores ml^{-1} alginate were suspended. This suspension was pumped slowly in to the 200-300 ml 4% $CaCl_2 \cdot 2H_2O$ solution by a peristaltic pump through a hypodermic needle. Droplets formed in the needle tip fall into the $CaCl_2$ solution, which was being stirred on magnetic stirrer, from approximately 10 cm and hardened immediately as Ca-alginate beads. Beads were approximately 2.5 mm in diameter. Beads were allowed to harden for 30-60 min and then were filtered on a mesh having a pore size of approximately 1mm in diameter, washed once with distilled water and suspended in culture media.

2.5. Culture Conditions

R. oryzae was cultivated in shake flasks and in bioreactor. In bioreactor continuous cultures with cell retention were performed. Unless otherwise stated mineral solution used in the cultivations were composed of (g l^{-1}); $(NH_4)_2SO_4$ (2), KH_2PO_4 (0.65), $MgSO_4 \cdot 7H_2O$ (0.25), $ZnSO_4 \cdot 7H_2O$ (0.05). Glucose, the carbon source of the fermentations, was supplied as glucose monohydrate.

2.5.1. Shake Flask Cultures

Lactate production medium was composed of mineral solution and varying amount of glucose. This medium was inoculated directly from the spore suspension to have final spore concentration of 1 x 10^3, $1x10^4$, $1x10^5$ or $1x10^6$ spores ml^{-1} of the culture medium. Fermentations were carried out in 250 ml cotton-plugged flasks containing 25, 50 or 100 ml culture medium in flask shaker with a shaking diameter of 12.5 mm (Aerotron, Infors AG) at 150 rpm and 35 ^oC. $CaCO_3$ powder was added intermittently in 0.25-0.50 g aliquots, when the media became clear due to the total utilization of $CaCO_3$. This ensured that pH was kept around 5-6. Media were sterilized in autoclave at 121 ^oC for 15 minutes. $CaCO_3$ powder was sterilized in oven at 100 ^oC overnight. Results reported for shake flask culture were generally average of three experiments.

2.5.2. Preculture for Fermenter

R. oryzae was pregrown in either mineral medium or in trypton-minerals (TM) medium.

For continuous cultures with cell retention, 50 ml alginate immobilized *R. oryzae* was pre-cultured in the fermenter in 1 l TM medium, containing of 10 g l^{-1} trypton and minerals at concentrations given above. pH of the medium was adjusted to 5.5 by concentrated HCl and was not controlled throughout the cultivation. pH increased to 6.5-7.0 in 24 h. Temperature was kept at 30 ^{o}C and stirring speed was 150 rpm. After 24-30 h stirring and aeration was shut down, so that beads were settled. Preculture medium was pumped out of the reactor using the exhaust line. Distilled water was added to wash the beads. After water was pumped out main culture medium was added.

Preculture for the glucose pulse experiments was conducted in 500 ml flask containing 100 ml medium containing minerals and 20 g l^{-1} glucose. Twenty ml alginate beads were grown for 28 h at 30 ^{o}C and 175 rpm.

For fed-batch cultures 50 ml alginate immobilized *R. oryzae* was pre-cultured in 2000 ml shake flask containing 500 ml mineral salts medium containing 50 g l^{-1} glucose and 7.5 g l^{-1} $CaCO_3$. Precultivation was carried out in flask shaker at 30 ^{o}C and 175 rpm for about 40 h. Beads were filtered through a mesh of pore size about 1 mm, washed with water and added to fermenter.

2.5.3. Preparation of the Fermenter

Fermentations were conducted in 3 l fermenter with a working volume of 2 l (Protem, Turkey). However, the culture volume of 1 l was used for this study. The fermenter was equipped with temperature, pH and dissolved O_2 controllers. Throughout the continuous cultures, pH was kept at 5.5 by automatic addition of 4-5 N KOH by a peristaltic pump connected to the pH controller. pH probe was

calibrated to pH 7 and pH 4 with commercial calibration buffers before sterilization of the fermenter. Cultures were aerated with 0.5-2 lpm air to keep dissolved oxygen above 25%, mostly around 40-50% of air saturation. Dissolved O_2 probe was calibrated to 0% by N_2 and to 100% by air before inoculation. Fermenter was sterilized at 121 ^oC for 20-30 minutes.

2.5.4. Continuous Culture with Cell Retention

After the preculture was pumped out of the fermenter and beads were washed once with water, 1 l mineral salts medium was added to the fermenter. Mineral salt concentrations were half of the values given for the shake flask cultures.. Feed medium composed of glucose at varying amounts and minerals was added for approximately 55 h. Mineral concentration of the feed medium was adjusted, so that carbon/nitrogen ration was 5 g g^{-1}. For every 4 g of glucose, (g l^{-1}) $(NH_4)_2SO_4$ (2), KH_2PO_4 (0.65), $MgSO_4 \cdot 7H_2O$ (0.25), $ZnSO_4 \cdot 7H_2O$ (0.05) was used. Feed medium was pumped using a speed adjustable peristaltic pump. Feeding was started at a low rate (around 0.6 ml min^{-1}) and after about 6 h feeding rate was increased to 2.24-3.05 ml/min. Culture volume was kept at 1 l by placing a exhaust pipe at the upper level of the medium (1 l). Liquid exceeding this level was pumped out by the exhaust pump running constantly (running faster than the feed pump). A mesh was attached to the pipe mouth, so that alginate beads did not leave the reactor in the exit stream. This set up was called continuous culture with cell retention, since there was a constant feed into and out of the reactor but the biomass stayed in the reactor. At the end of the fermentation, beads and free mycelium were filtered through cheese cloth and washed several times with water and dried at 100 ^oC overnight.

Average medium glucose concentration was estimated from the measured glucose concentration throughout the experiment. The total lactate and ethanol produced was calculated by calculating the areas under the lactate and ethanol curves in the time vs. concentration graphs multiplied by the feeding rate. Results were used to calculate the product yields. Product yield was defined as the total product (lactate

or ethanol) produced per total glucose consumed (g product g glucose^{-1}). Biomass yield was the difference of the biomass measured at the end and at the start of the continuous culture per total glucose consumed. The amount of glucose consumed was calculated by subtracting the unused glucose from the amount of glucose added in the feed. Unused glucose was calculated similar to the total product concentration.

2.5.5. Fed-batch Culture

Alginate beads were transferred to reactor containing one liter mineral solution. Feed was composed of mineral solutions and 20 g l^{-1} glucose. Feeding started at a rate of 3.4 ml h^{-1} and rate was increased to 17.1 ml h^{-1} in 72 h.

2.6. Analytical Methods

One to two ml of fermentation medium either from the shake flasks or the fermenter were sampled and centrifuged at 12000 g for 5 min. Pellet was discarded and the supernatants were kept at -18 ^{o}C until used. Samples were thawed at 4 ^{o}C, diluted with HPLC eluent and analyzed for glucose, lactate, and ethanol by HPLC (LKB, USA) using an organic acid analysis column, which using mainly ion – exclusion mechanism (Phenomenex, Torrance, CA, USA), and a differential refractometer (Schambeck RI2000, Bad Honnef, Germany) (See Appendix B for a sample chromatogram). The column contained sulfonated styrene-divinylbenzene spheresin 8% cross-link form as well as hydrogen ionic form. The column was kept at 50-65 ^{o}C and was eluted with 5 mM H_2SO_4. The detector cell was kept at 35 ^{o}C. Glucose, lactate and ethanol standard solutions of known concentrations were used for calibration. Signal from the detector was processed by Chromasimple data acquisition software (Dizge Analitik, Turkey). Lactate was also analyzed with a lactate analyzer (YSI 1500 Sport Lactate Analyzer, Yellow Spring, USA). The immobilized L-lactate oxidase converted L-lactate to hydrogen peroxide, which was oxidized at the platinum electrode producing electrons. The resulting current was proportional to L-lactate. Glucose concentration of the continuous culture was determined by an enzymatic glucose kit (Infinity ^{TM},

Thermo, Victoria, Australia). Glucose oxidase in the reaction mixture converted glucose to gluconic acid and produced hydrogen peroxide. Peroxide reacted with 4-hydrobenzoic acid and 4-aminoantipyrine in the presence of peroxidase forming a quinoneimine dye. The intensity of the color formed was proportional to the glucose concentration and measured at 500 nm.

At the end of the fermentations, mycelia were collected on a metal mash having a pore size approximately 1 mm. Unused $CaCO_3$ remaining on the mycelia from shake flask cultures was dissolved by HCl. After washing several times with distilled water, dry cell weight was determined by drying the mycelia overnight at 100 ^{o}C.

2.6.1. Extraction of Proteins

Free mycelia from shake flask cultures or alginate immobilized mycelia was filtered and washed twice with distilled water and once with the extraction buffer (100 mM phosphate buffer at pH 7, containing 1 mM EDTA, 1.4 mM β-mercaptoethanol and 1 mM PMSF). Samples were dried between filter papers and squeezed gently to form a thin layer of biomass. Samples were packed in aluminum foil and were frozen in liquid nitrogen and kept at –80 ^{o}C until used. Frozen cells were brought to powder form by Mikro-D95 dismembrator (Sartorius). A thin biomass layer and a steel ball were put in PTFE container, which were cooled in liquid nitrogen. This container was shaken for 30-60 seconds at 2000 rpm. This procedure crushed the mycelia to a powder form, which was then suspended in cold extraction buffer. Cell debris was removed by centrifugation at 12500 g for 30 min at 4 ^{o}C. Supernatant (crude extract) was used for enzyme assays.

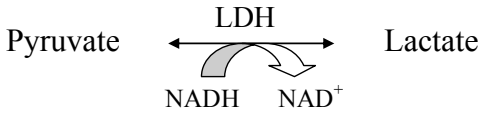
2.6.2. Enzyme Assays

All enzyme activities were measured at 30 ^{o}C by monitoring the oxidation of NADH or reduction of NAD^{+} at 340 nm in spectrophotometer (Shimadzu UV-

1202, Japan). Crude extract concentration in the assay mixture were adjusted to ensure that change in absorbance followed first order kinetics at least for 4 min and that change in absorbance was proportional to the amount of crude extract added. One unit of enzyme activity was defined as the amount of enzyme required to catalyze the oxidation of 1 μmole of NADH to NAD^+ or reduction of 1 μmole of NAD^+ to NADH in 1 min under specified conditions. Extinction coefficient of NADH was taken as 6.22 mM^{-1} cm^{-1}. Assays were done in 1.4 ml special glass cells (Hellma, Germany) having a light path of 1 cm and assay volume was 1.2 ml for all assays. Activities were reported as specific activities, which were defined as the unit of enzyme per mg of protein in the crude extract. Protein was determined by Lowry method (42) with bovine serum albumin as the standard. Crude extracts generally contained 1-2 mg protein ml^{-1}.

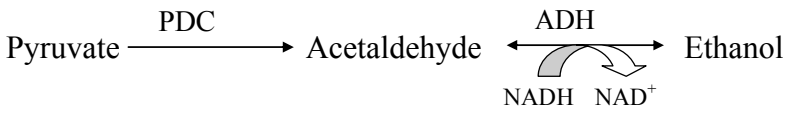
2.6.2.1. Lactate Dehydrogenase Assay

LDH was assayed in 100 mM bis-Tris-propane buffer (pH 6.8) containing 0.175 mM NADH (68). Reaction was started by adding 4mM pyruvate.
When *R. oryzae* was grown at low glucose concentrations, Tris buffer (pH=7.5) was used. Pyruvate and NADH concentrations were the same as above.



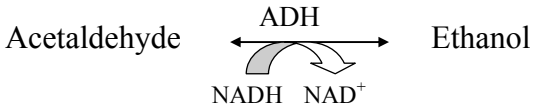
2.6.2.2. Pyruvate Decarboxylase Assay

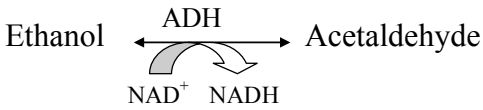
PDC was assayed in 100 mM MES buffer (pH 6.2) containing 0.175 mM NADH, 2mM $MgCl_2$, 1mM TPP, 0.04 Units yeast ADH μl^{-1} (69). ADH was used as the coupling enzyme. Reaction was started by adding 4 mM pyruvate. Interfering LDH activity was inhibited by adding 25 mM oxamate.



2.6.2.3. Alcohol Dehydrogenase Assay

ADH assay was optimized in our laboratory and the reaction mixture contained 100 mM phosphate buffer (pH 6.3) 0.15 mM NADH. Reaction was started by adding 5mM acetaldehyde. When *R. oryzae* was grown at low glucose concentrations, ADH was assayed in the other direction, reduction of ethanol to acetaldehyde, in glycine buffer (pH 9.0), containing 100 mM ethanol and 1 mM NAD.





CHAPTER 3

RESULTS AND DISCUSSION

3.1. Shake Flask Cultures

3.1.1. Effect of Inoculum Size and Working Volume on Lactic Acid and Ethanol Production and Morphology

Previous studies in our laboratory and the preliminary experiments of this study, as well as the literature, showed that inoculum size had a drastic effect on the fungal physiology and morphology. Our aim was to quantify the effect through extracellular metabolites and examine the macro-morphology. Previous reports also showed that lactate production by *Rhizopus oryzae* needs vigorous aeration of the cultures. Therefore, we conducted fermentation runs with different inoculum concentrations and working volumes in shake flasks.

Lactic acid and ethanol and biomass productions by *R. oryzae* were investigated in 250 ml shake flask cultures containing 25, 50 or 100 ml culture medium inoculated with $1x10^3$, $1x10^4$, $1x10^5$ or $1x10^6$ spores ml^{-1}. Cultures, which had 100 g l^{-1} initial glucose concentration, were inoculated directly from spore suspension. The final concentrations of lactic acid, ethanol, and biomass (dry cell weight) are shown in Figure 3.1, Figure 3.2 and Figure 3.3, respectively. Fermentations were ended when all glucose was utilized. Final volumes were recorded and metabolite concentrations were corrected for the initial volumes.

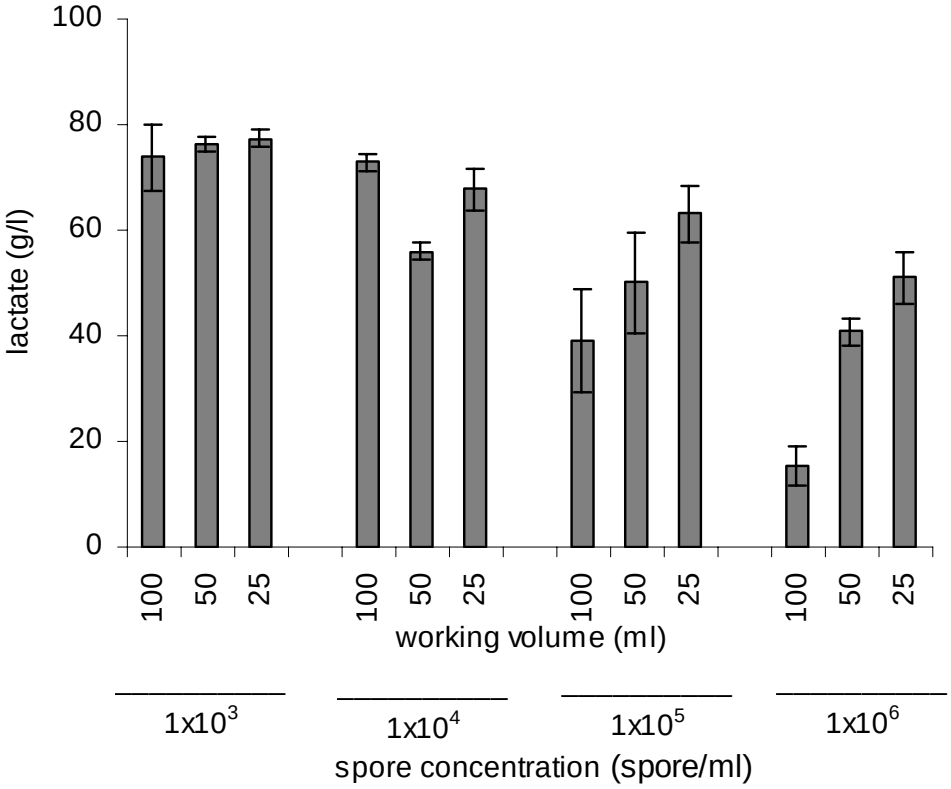


Figure 3.1. Effect of spore concentration and working volume on lactate production by *R. oryzae*. (S_o=100 g l^{-1})

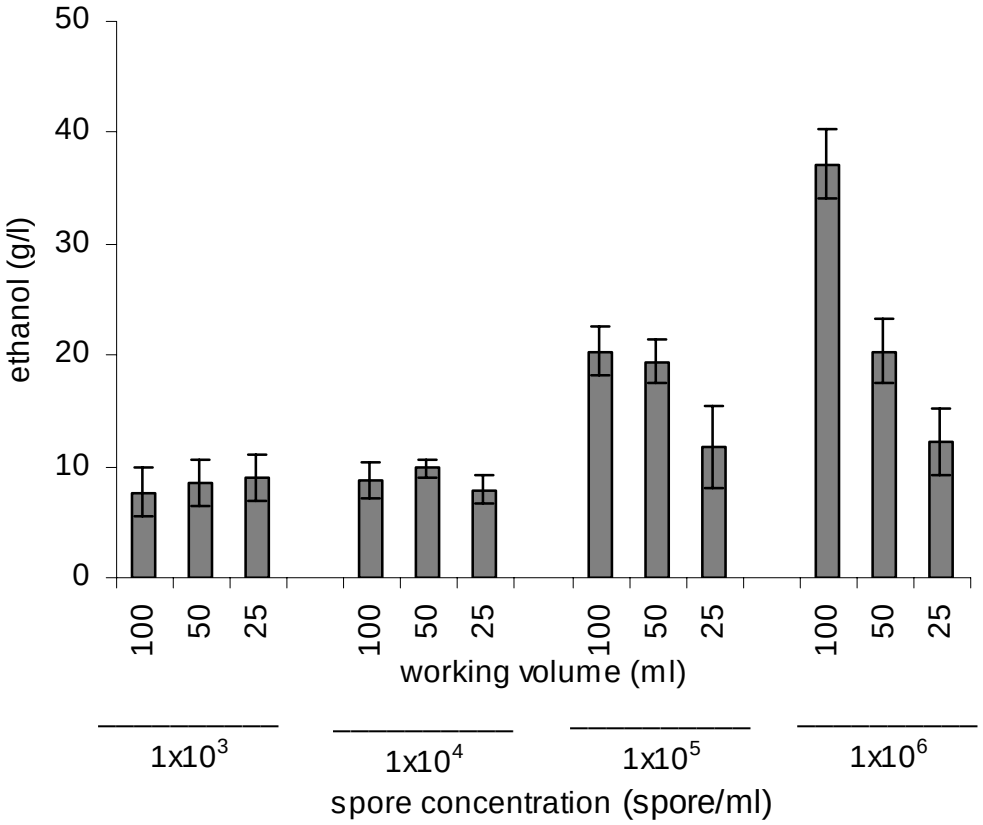


Figure 3.2. Effect of spore concentration and working volume on ethanol production by *R. oryzae*. (S_o=100 g l^{-1})

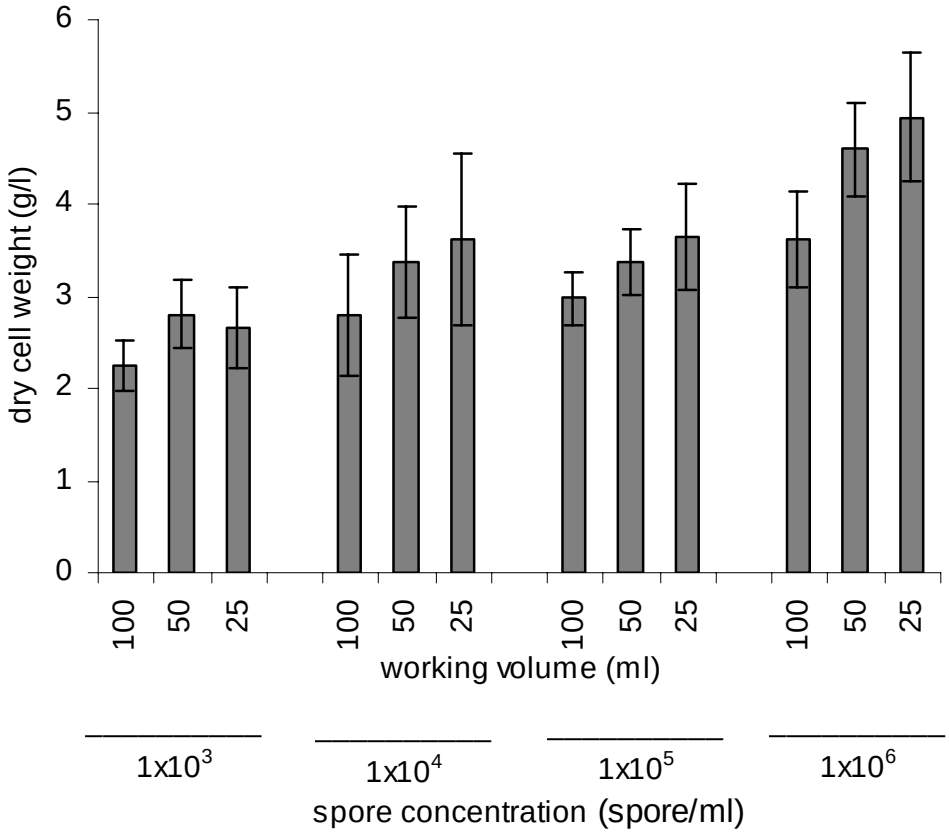


Figure 3.3. Effect of spore concentration and working volume on biomass production by *R. oryzae*. (S_o=100 g l^{-1})

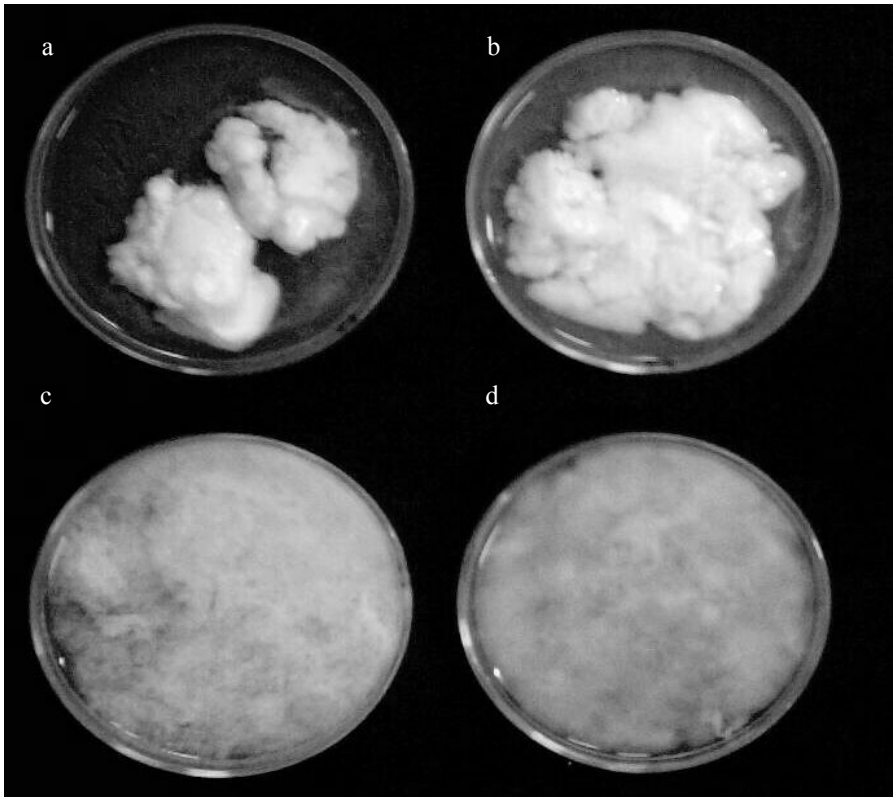


Figure 3.4. Effect of inoculum size on morphology in cultures with 100 ml working volumes. Photographs were taken at the end of the fermentations. Biomass was put in Petri plates to take the photographs.
a. $1x10^3$, b. $1x10^4$, c. $1x10^5$, d. $1x10^6$ spores ml^{-1}

In the cultures having high inoculum ($1x10^5$ and $1x10^6$ spores ml^{-1}) fermentations lasted about 48 h, while fermentation time of the cultures having low inoculum sizes ($1x10^3$ and $1x10^4$ spores ml^{-1}) extended to 64 h. Generally, lactic acid levels were decreased with increasing inoculum size, while ethanol and biomass levels were increased. The increase in the working volume induced higher ethanol production and lower lactic acid and biomass. Changing the working volume influenced the cultures which had high inoculum size ($1x10^5$, $1x10^6$), more drastically. The highest lactic acid (78 g l^{-1}) and lowest ethanol (8 g l^{-1}) and biomass (2.3 g l^{-1}) levels were obtained with lowest inoculated spore concentration ($1x10^3$

spores ml^{-1}). At this inoculum size, working volume did not have an effect on the metabolite concentrations, while biomass concentrations were higher in 25 and 50 ml cultures (2.8 and 2.7 g l^{-1}, respectively). The lowest lactic acid (15.3 g l^{-1}) and highest ethanol (37.2 g l^{-1}) production were obtained with $1x10^6$ spores ml^{-1} inoculum and 100 ml culture volume, which may have had the lowest dissolved O_2 concentration. The maximum biomass concentration (4.9 g l^{-1}) was obtained with high inoculum ($1x10^6$ spores ml^{-1}) and low volume (25 ml). Highest lactate productivity values were around 1.1-1.2 g l^{-1} h^{-1}, which is obtained at low inoculum size. Ethanol productivity was maximized by using high inoculum and high working volume (0.77 g l^{-1} h^{-1}). Mass balance showed that 84-100 % of the initial glucose was accounted for biomass, lactate and ethanol (See Appendix C for sample mass balance). The glucose that not accounted may have been used for production of other trace metabolites.

High lactic acid concentration (72.8 g l^{-1}) obtained in 100 ml culture having a spore concentration of $1x10^4$ spores ml^{-1} was unexpected since this value was higher than that of the lower culture volumes. At this inoculum level aeration did not have a significant effect on the ethanol concentrations, which were comparable to that of obtained by $1x10^3$ spores ml^{-1} inoculum.

Growth form varied from clumps to filamentous mycelia. In Figure 3.4 the morphologies in the 100 ml cultures are shown. Inoculum concentrations of $1x10^3$ and $1x10^4$ spores ml^{-1} induced one big tightly packed clump composed of both the mycelia and $CaCO_3$ particles (Figure 3.4 a and b). This form left a clear medium around it. In the cultures having higher inocula *R. oryzae* grew as filamentous mycelia (Figure 3.4 c and d). In 50 ml cultures morphologies were similar to that of 100 ml cultures. In the flasks having 25 ml culture medium, however, the morphologies were harder to define. Since the liquid levels in the flasks were low, most of the culture skimmed over the walls. Therefore, biomass together with $CaCO_3$ adhered to the walls of the flask. In most of the cultures, these masses fell into the medium and formed a ribbon like structure.

High lactate yield obtained at low inoculum sizes is comparable to other studies. Lactic acid yields in previous reports ranged from 65 to 90 % (80). Although *R. oryzae* is generally known to produce lactate, the results showed the potential of this fungus for ethanol production. The ethanol yield was higher than the *Aspergillus* and *Rhizopus* species screened by Skory et al. (71). Soccol et al. (75) obtained similar ethanol yield with *R. oryzae* NRRL 393 by sealing the flasks to supply O_2 limited conditions. In this study, all fermentations were conducted under aerobic conditions in cotton plugged shake flask. Therefore, decreasing the aeration may have increased the ethanol concentration (Fig. 3.2).

The inoculated spore concentration and the working volume were shown to influence the morphology and the lactate and ethanol productions in the shake flask cultures of *R. oryzae* (Fig. 3.1, Fig. 3.2, and Fig. 3.4). Thus, inoculum size and working volume (aeration) can be adjusted for desired applications, namely lactate production, ethanol production or biomass related applications. For example, when one intends to produce lactic acid, $1x10^3$ or $1x10^4$ spores ml^{-1} are the most suitable inoculum sizes, while inoculum size of $1x10^6$ spores ml^{-1} and high working volume should be used to produce ethanol. Inoculum size of $1x10^6$ spores ml^{-1} and low working volume could yield high biomass concentration.

In this study higher lactate and lower ethanol levels were obtained by decreasing the inoculated spore concentration, under which conditions *R. oryzae* grew as clumps (85). This is contradictory to the results of Yin et al. (85) and Bai et al.(2). Both groups generally obtained higher lactate and biomass levels as they increased the inoculated spore concentration. Lactic acid concentration was maximized when the *R. oryzae* grew as pellets of definite sizes, and congealed or flocculated mycelia resulted in lower lactic acid yield. Pellets were obtained with inoculum size of $1x10^6$ spores ml^{-1} (2) or $2x10^6$ spores ml^{-1} (85). They concluded that mass (both nutrient and oxygen) transfer was better in the pellets than in the big mycelial masses. Therefore, we propose that the relation between the morphology and the physiology of *R. oryzae* is complicated and should be studied in more detail.

Lactic acid production has been reported to increase with increasing aeration (80). Aeration may have been changed in the flask cultures by changing the working volume. Each aeration level was investigated at each inoculum size and the lactate, ethanol and biomass levels were influenced differently at different inoculum sizes. Increasing aeration yielded higher lactate and biomass and lower ethanol. The effect of aeration was negligible at $1x10^3$ spore ml^{-1} inoculum size and become more considerable as the inoculum size increased. Available O_2 may have been sufficient in 100 ml cultures in the low inoculum size cultures; therefore, increasing aeration may have not increased the lactate production (Fig. 3.1). One hundred milliliters of working volume in 250 ml flask is generally considered too much for an aerobic organism. In this study, however, at low inoculum sizes working volume could be increased as high as 100 ml without a decrease in lactate production. It should be noted that the dissolved O_2 levels were not measured and they should have been different for each inoculum size and working volume.

3.1.2. Kinetics of High Lactate Producing Fermentation

In this part of the study, biomass concentration of the organism was followed under the conditions yielding high lactate production, as well as the lactate and ethanol levels. For this purpose several 250 ml flasks containing 50 ml medium were inoculated at same time and for each time point one flask was sacrificed. Initial glucose concentrations of the cultures were 100 g l^{-1}. Metabolite levels and dry cell weight were determined (Figure 3.5). Biomass concentration followed a parallel trend with metabolites. Approximately 2.5 g l^{-1} biomass and 70 g l^{-1} lactate and 15 g l^{-1} ethanol were obtained at the end of the fermentation. Between 42 and 53 h of the fermentation, glucose consumption and lactate and ethanol production was fastest. Glucose consumption rate was 4.4 g l^{-1} h^{-1}, lactate and ethanol production rates were 3.7 and 0.3 g l^{-1} h^{-1}, respectively. Biomass production rate was around 0.15 g l^{-1} h^{-1} in this period. Overall lactate production rate was around 1 g l^{-1} h^{-1} and lactate yield (Y_{SX}) was 0.67, g g glucose^{-1}. Under these conditions, high lactate concentrations could be obtained. However, due to the low amount of inoculum fast utilization of glucose started after 35 h of

fermentation. Therefore, although lactate concentration was high, productivity values were low. Using seed cultures, lag time of the fermentations can be shortened.

Since *R. oryzae* grew as clumps or intertwined filamentous mycelia, measuring biomass concentration in a single culture was impossible. Therefore, for each point, one or more flask was sacrificed and DCW was measured. It can be considered as a primitive way to obtain a growth curve, however it was the only way to measure biomass concentration and relate lactate and ethanol production to biomass concentration. Since lactate and ethanol are primary metabolites, i.e. they are related to growth of the organism, biomass curve followed a parallel pattern to lactate and ethanol.

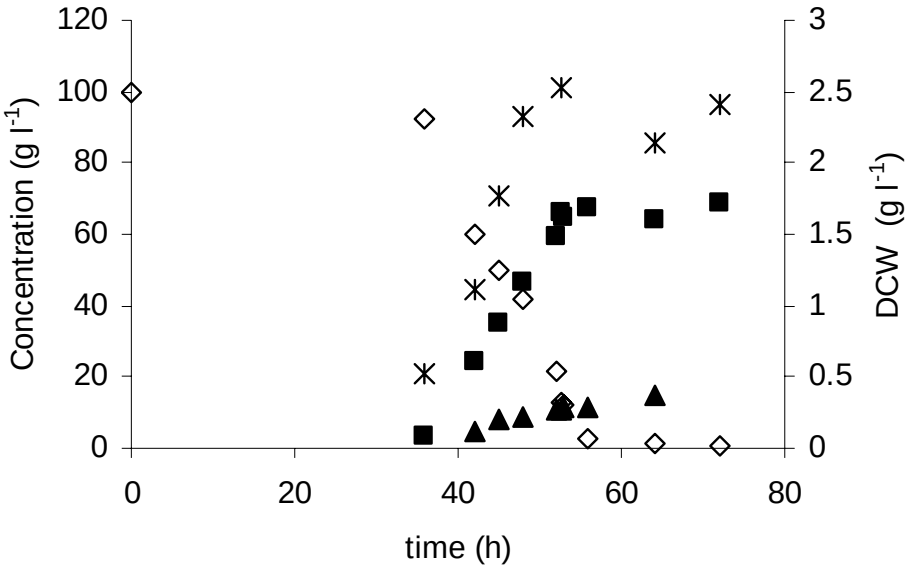


Figure 3.5. Time course of glucose consumption (◊), lactate (■) and ethanol (▲) production and dry cell weight (x) in low inoculum size ($1x10^3$ spores ml^{-1}) culture with 50 ml working volume

3.1.3. Time Course of Enzymatic Activities

In the above sections, different physiologies of *R. oryzae* were observed depending on the culture conditions. Lactate and ethanol production was influenced significantly. Since these two metabolites are originated from pyruvate, the enzymes of the pyruvate branch point that produces lactate and ethanol may play a role in this different physiology of the organism. In an effort to quantify the levels of these enzymes, their specific activities were followed throughout the fermentations. In the beginning, fermentations were conducted as described in the previous section; several flasks were inoculated at the same time and one was sacrificed for each data point and metabolites and enzymatic activities were determined. However, especially enzymatic activities of different flasks showed great variance, most probably due to morphological differences. A general trend for each enzyme could be observed but there were some extreme values in the data set. Therefore, it was decided to follow the enzymatic activities of samples from the same flask. This limited the number of samples that could be taken from the flasks, since a certain amount of biomass should be taken for the extraction procedure. Activities were determined at four different times of the fermentations.

Lactate and ethanol levels were followed as well as LDH, PDC and ADH specific activities in three parallel flasks. Conditions were adjusted to maximize lactate in one culture and ethanol in the other. Former was inoculated with $1x10^3$ spores ml^{-1} and the latter one with $1x10^6$ spores ml^{-1}. Both fermentations were carried out in 250 ml flask with 100 ml working volume. In lactate dominant culture, total utilization of glucose took approximately 66 hours and 75 g l^{-1} lactate and 9.3 g l^{-1} ethanol were produced (Figure 3.6 A). In ethanol dominant fermentation, glucose was depleted in 43 h and 36 g l^{-1} ethanol and 16 g l^{-1} lactate were produced (Figure 3.6 B). In the period of fast glucose consumption, the consumption rate was around 4.4 g l^{-1} h^{-1} in both cases, however with low inoculum size glucose utilization started later. Lactate and ethanol production was in parallel to glucose

consumption, but their production rates were different and affected by the conditions studied.

Enzyme activities generally increased with time and decreased as glucose was depleted (Figure 3.7). Time course of three enzyme activities generally followed the same pattern. In lactate dominant culture, maximum LDH activity was approximately 8.5 times higher than in ethanol dominant one. PDC and ADH specific activities were 1.5 and 1.8 times higher in ethanol dominant fermentation, respectively. They were not influenced as much as LDH under the conditions studied. The amount of LDH may have determined the fate of pyruvate to ethanol or lactate. PDC is involved in supplying cytosolic acetyl-CoA, which is used for the synthesis of lipids and some amino acids. Therefore, its high activity in lactate dominant cultures may be explained by this role of the enzyme. A portion of the acetaldehyde produced by the action of PDC can be oxidized to acetate by acetaldehyde dehydrogenase. Acetate, in turn, can be converted to acetyl-CoA by acetyl-CoA synthetase, by using ATP. However, activity of ADH in lactate dominant cultures can not be explained by this, since it is not involved in cytosolic production of acetyl-CoA.

Specific activities of the enzymes agreed well with the dominance of lactate or ethanol, i.e. specific activity of LDH was high in high lactate yielding fermentation, whereas PDC and ADH exhibited higher specific activities in high ethanol yielding fermentation. Time course trends observed for *R. oryzae* LDH activity were similar to previous results ((57; 68; 70)). Skory (69) observed increased transcript of both *pdcA* and *pdcB*, and also increased enzymatic activities and increased ethanol in anoxic *R. oryzae* cultures compared to aerobic ones. ADH was also shown to exhibit higher activities in anaerobic *R. oryzae* cultures (72). To our knowledge, this is the first study that compared the actions of these three enzymes.

The enzyme activity data was used to estimate the flux through lactate and ethanol producing pathways (See Appendix D for calculations). LDH was

assumed to obey Michealis-Menten kinetics (57), while PDC was shown to obey the Hill equation (1). ADH was excluded from this calculation, since its *in vitro* activity was much higher than PDC. *In vivo* rate of the lactate and ethanol productions were estimated using the measured activities of the enzymes and K_m values taken from the literature. Rates were calculated for a range of intracellular pyruvate concentrations using Michaelis-Menten and Hill kinetic expression. The rates calculated were compared to the experimental data. In lactate producing batch culture measured rates of lactate and ethanol were obtained at pyruvate concentrations of 0.16 and 0.67 mM, respectively. In ethanol producing batch culture, measured rates were obtained at 0.26 and 0.88 mM pyruvate for lactate and ethanol production rates, respectively. In the literature, different intracellular concentrations of pyruvate were reported (0.04 mM for *Aspergillus niger* (64), 2.2-4.9 mM for *Monascus rubber* (21), 7-10 mM (18; 20)**,** 1.85 mM (81) for *S. cerevisiae*). The pyruvate concentrations estimated using the Michaelis-Menten and the Hill equations were in the reasonable range for an organism. In both of the cases, measured fluxes of lactate and ethanol could be described by different pyruvate concentrations. Pyruvate needed was lower for the flux through lactate than flux through ethanol. Reason for that could be the insufficiency of *in vitro* determination of the enzyme activities to describe the *in vivo* activity. The measured activities may have either overestimated or underestimated the *in vivo* activity. The Michaelis-Menten and the Hill type kinetics may have not been sufficient to describe the rate of the reactions. In addition to these, several assumptions were made in calculations, which could cause deviations from the actual values.

In another approach, kinetic parameters, K_m and V_{max}, were calculated using the measured rates of the lactate and ethanol productions (See Appendix D for equations). In order to calculate K_m values, measured enzyme activities were used as the V_{max}. On the other hand, to estimate V_{max} values, K_m values from the literature were used. For example, assuming an intracellular pyruvate concentration of 1 mM, K_m value of LDH of 3.81 mM and of PDC 9.43 mM would yield the observed rate of lactate and ethanol productions in lactate

producing culture. Using the K_m values reported in the literature required maximum activity of LDH and PDC were calculated as 0.80 and 0.65 U mg protein^{-1}, respectively. Similarly, in ethanol producing culture, at a pyruvate concentration of 1mM, K_m values of 2.29 and 5.47 mM of LDH and PDC, respectively, were approximated. Required maximum activity values would be 0.15 and 1.45 U mg protein^{-1} for LDH and PDC, respectively. Calculated values for K_m of LDH for pyruvate was 5-7 fold higher than the literature value, while K_m of PDC was not higher more than 2 fold. At a pyruvate concentration of 2 mM K_m values calculated for both enzymes in both cases were very high, indicating that intracellular pyruvate concentration should be below 2mM. Required LDH activity at a pyruvate concentration of 1 mM was lower than the experimental activity for both lactate and ethanol producing cultures. Calculated activities of the PDC were not as different from the experimental activity as of LDH. This could indicate that PDC assay was more reliable than LDH assay. PDC may have interfered in the LDH activity measurement. However, for a more reliable discussion of the results obtained by kinetic analysis, the concentration of the intracellular metabolites, i.e. pyruvate in this case, should be known.

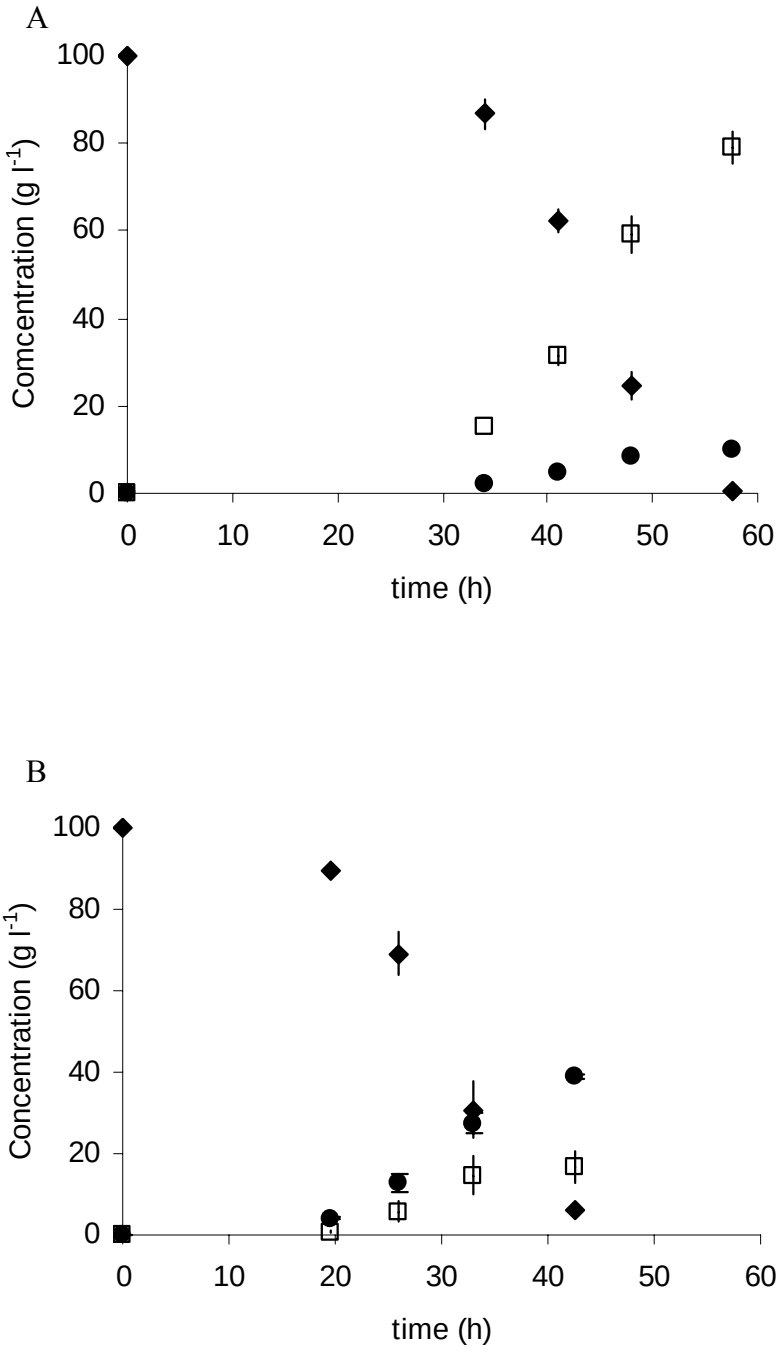


Figure 3.6. Glucose consumption (♦) and lactate (□) and ethanol (●) productions in cultures inoculated with A: $1x10^3$ spores ml^{-1}, B: $1x10^6$ spores ml^{-1}. Error bars indicate standard deviations. (Culture volume=100 ml, S_o=100 g l^{-1})

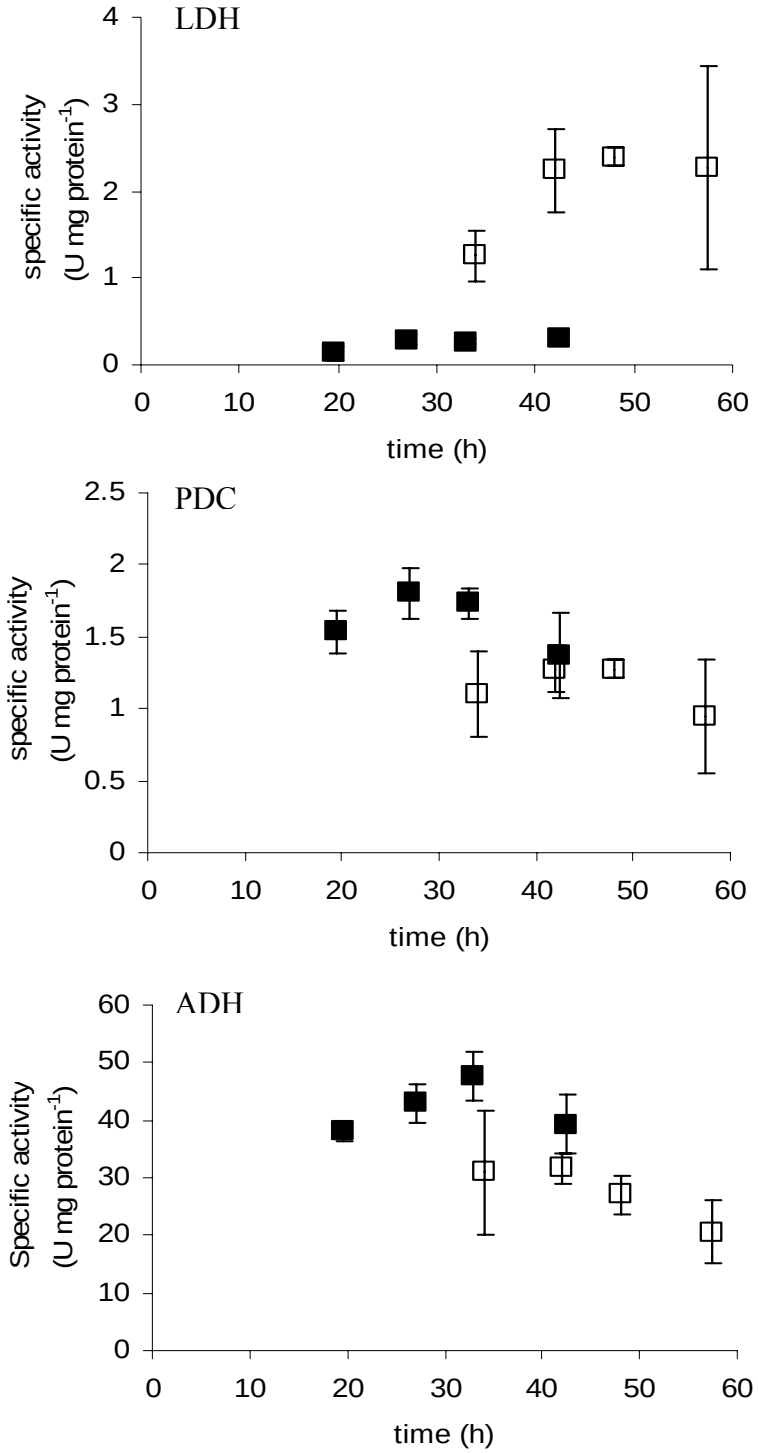


Figure 3.7. Comparison of specific activities of lactate dehydrogenase (LDH), pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) in cultures inoculated with $1x10^3$ (□) or $1x10^6$ (■) spores ml^{-1}. Error bars indicate standard deviations. (Culture volume=100 ml, S_o=100 g l^{-1}

3.1.4. Effect of Glucose Concentration

R. oryzae was grown in 250 ml shaking flasks with a working volume of 50 ml under initial glucose concentrations (S_o) ranging from 0.5 to 100 g l^{-1}. Flasks were inoculated with $1x10^5$ spores ml^{-1}. L-lactate, ethanol and biomass concentrations were measured at the end of the fermentations when glucose was exhausted. Generally, biomass yield decreased and L-lactate and ethanol yields increased as S_o increased (Figure 3.8). At two lowest S_o studied (0.5 and 1 g l^{-1}) high biomass yields were obtained (0.39 and 0.31 g DCW g $glucose^{-1}$, respectively). On the contrary, at these glucose concentrations lactate and ethanol yields were very low. When S_o was increased to 10 g l^{-1} biomass yield decreased and product yields increased drastically. Lactate yield increased from 0.34 to 0.50 and ethanol yield increased from 0.12 to 0.19 g g $glucose^{-1}$ as S_o was increased from 10 to 100 g l^{-1}. Biomass yield was around 0.20 g DCW g $glucose^{-1}$ at S_o of 10 g l^{-1} and decreased to 0.034 at S_o of 100 g l^{-1}. Thus, lactate and ethanol produced per DCW was increased as S_o was increased (Figure 3.9).

Initial glucose concentration had a considerable influence on the physiology of *R. oryzae*. Low biomass yield and high product yield at high glucose concentrations can be explained by overflow metabolism at pyruvate branch. High S_o values may have yielded high growth rates, under which conditions pyruvate may have accumulated and may have been utilized for production of lactate and ethanol. Pyruvate accumulation can be explained by the low capacity of the respiratory system of the organism. At high S_o values nitrogen may have been limiting for the growth and more glucose was diverted to lactate and ethanol, yielding high product per biomass yield. For lactic acid production, therefore, glucose (or other carbohydrates) concentration should be kept high. C/N ratio may be determining in this phenomenon, so that it needs further studies. In his study on LDH, Pritchard (57) observed similar biomass yields with initial glucose concentrations between 2.5 and 10 g l^{-1} and the yield dropped significantly as the S_o was increased to 20 g l^{-1}. It should be noted that there was glutamate in his medium formulation, which may have influenced the growth.

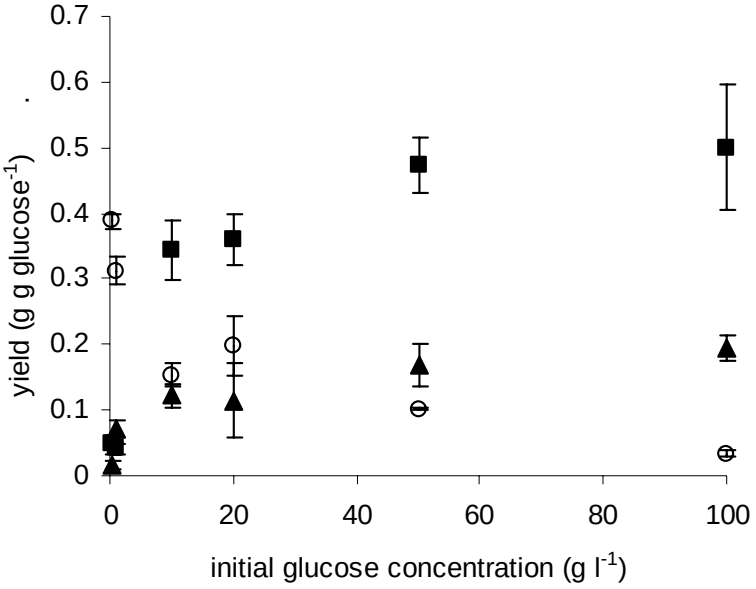


Figure 3.8. Effect of initial glucose concentration on lactate (■), ethanol (▲) and biomass yields (○). (g product per g glucose consumed). Error bars indicate standard deviations (Inoculum size=1x10^5 spores ml^{-1}, culture volume=50 ml, S_o=100 g l^{-1}

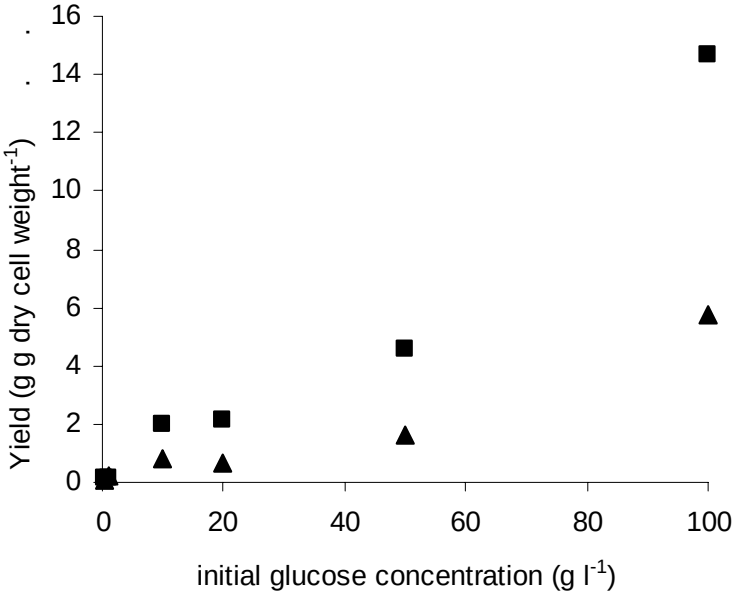


Figure 3.9. Effect of initial glucose concentration on yield values (g product per g DCW). lactate yield on biomass (■), ethanol yield on biomass (▲).(Inoculum size=1x10^5 spores ml^{-1}, culture volume=50 ml, S_o=100 g l^{-1}

Physiology of *R. oryzae* at low glucose concentration was further investigated by intermittent addition of small portions of glucose, so that glucose was kept below certain concentrations. Glucose was kept below 0.40 l^{-1} in one culture and 0.72 g l^{-1} in the other. When C_G dropped below about 0.1 g l^{-1}, glucose was added to attain a C_G of either concentration (Figure 3.10). In 12 h. total of 2.90 and 5.63 g glucose was added to former and latter cultures, respectively. Lactate and biomass concentrations and yield values are shown in Table 3.1. Lactate yields were lower than biomass yields as expected, however, Y_{SL} values were higher and Y_{SX} values were below those were obtained in the experiments mentioned above in this section.

Table 3.1. Lactic acid and biomass yields of *R. oryzae* grown on low concentrations of glucose in shake flasks

Glucose concentration, C_G (g l^{-1})	Glucose utilized (g l^{-1})	Lactic Acid, C_L (g l^{-1})	Lactate Yield, Y_{SL} (g g^{-1})	Dry Weight, C_X (g l^{-1})	Biomass Yield, Y_{SX} (g g^{-1})
Glu ≤ 0.40	2.90	0.44	0.151	0.878	0.26
Glu ≤ 0.72	5.63	1.06	0.190	1.932	0.31

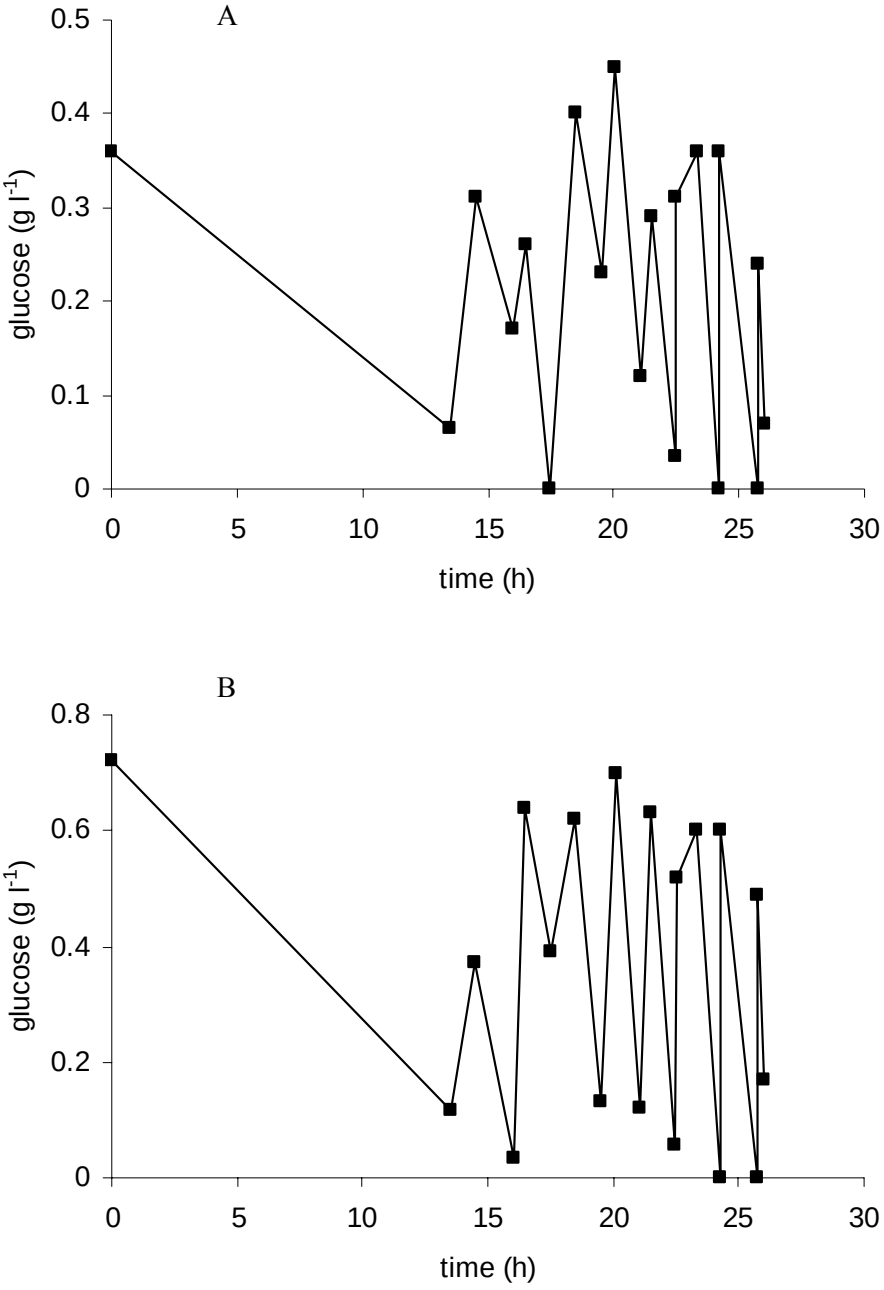


Figure 3.10. Change in glucose concentration (Inoculum size=1x10^5 spores ml^{-1}, culture volume=50 ml

3.2. Fermenter Cultures with Immobilized Cells

In order to elucidate the effect of glucose concentration on physiology, experiments under controlled conditions were needed. In bioreactor, pH and dissolved O_2 concentration could be measured and controlled as well as temperature and agitation. Apart from those, bioreactor allowed us to carry out continuous culture (See Material ands Methods). Having adhered the inside elements (including the probes) of the reactors, *R. oryzae* was hard to cultivate as free mycelium (Figure 3.11). Therefore, we immobilized the spores in alginate beads.

Figure 3.11 Pictures of *R. oryzae* in bioreactor. Left: Alginate immobilized mycelia, Right: Free mycelia attached on the impeller, probes and sparger.

3.2.1. Continuous Cultures with Cell Retention

Alginate immobilized *R. oryzae* was cultured continuously by feeding glucose at varying concentrations (2.5-20 g l^{-1}). The organism was first grown in batch mode

in Trypton-Mineral Salts (TM) medium in the reactor. In the beginning, batch culture was carried out in the medium as proposed by Skory (69). In that study, the medium containing glycerol and trypton was shown not to support synthesis of lactate and ethanol. However, in this study, in the same medium *R. oryzae* did not use glycerol in two days (Results not shown). Therefore, glycerol was excluded and grew the fungus in a medium containing only trypton and minerals. Using such a medium provided substantial amount of fungal biomass, in which PDC and LDH showed little or no activity (data not shown). After 24 h, this medium was pumped out and beads were washed once with distilled water. Fresh mineral medium containing no glucose was added and glucose feed started. In each culture, single feed glucose concentration ($C_{G,\,feed}$) was used. Different $C_{G,\,feed}$ yielded different reactor glucose concentrations ($C_{G,\,reactor}$), which allowed us to investigate the effect of glucose concentration on physiology of the organism. Feeding rate was adjusted between 2.5-3.0 ml min^{-1}, so that at least four volume changes were guarantied in two days. Fermentation kinetics of the cultures are shown in Figure 3.11. 1-5.

In Table 3.2 total amount of glucose fed to the reactor and total amount of lactate and ethanol drawn out from the reactor in the effluent stream are shown. Lactate and ethanol levels of the cultures were frequently measured throughout the cultures. Total amounts of the metabolites produced throughout the continuous culture were calculated by integrating the concentration curves. Yield values were calculated by dividing the total amount of lactate, ethanol and biomass produced, by the total amount of glucose utilized. Amount of glucose utilized was the difference between the amounts of glucose fed to the reactor and unused glucose drawn out in the effluent stream.

As ($C_{G,\,feed}$) was increased, $C_{G,\,reactor}$ and lactate yield (Y_{SL}) increased and biomass yield (Y_{SX}) decreased. Ethanol yield (Y_{SE}) was first increased, but then decreased upon further increase in $C_{G,\,feed}$. Comparing the lactate and ethanol yields, one can see that ethanol was predominant at low glucose concentrations (Table 3.2). At higher concentrations lactate yield was higher than ethanol yield. Lactate yield

showed a sharp increase as glucose concentration increased to 0.2-0.3 g l^{-1}. Further increase to 0.5-0.6 g l^{-1} supported higher lactate yield than ethanol yield. At $C_{G,reactor}$ of 0.03-0.05 g l^{-1}, lactate yield was very low, but ethanol yield was considerably high. Biomass yield was influence by the glucose concentration inversely. As glucose concentration was increased more glucose is fermented to lactate and ethanol, and consequently less is used for the growth. Higher biomass yields were observed at lower $C_{G,\ reactor}$, however, values were much below than the expected maximum yield (around 0.5 g g glucose^{-1} for *S. cerevisiae*). The biomass concentration was estimated by the difference between the weight of the beads before and after the cultures. Portion of the Ca-alginate forming the beads may have dissolved in the fermentation media. Therefore, biomass concentration determined could be a little bit less than the actual value.

Carbon balance was determined for the cultures. Carbon accounted by lactate, ethanol and biomass were about 0.7-0.8 of the carbon added to the culture as glucose. This may have been due to the other metabolites produced by *R. oryzae*, such as glycerol and fumaric acid. Some carbon could have been lost due to evaporation of ethanol in the off gas.

The distribution of pyruvate among the fermentative and respiratory pathways was different at different glucose availability. Kinetics and the levels of the pyruvate branch point enzymes may have played role as was shown in the shake flask cultures. Specific activity measurements of LDH, PDC and ADH showed that these three enzymes were influenced by the medium glucose concentration (Table 3.3). Generally, LDH and PDC increased as $C_{G,\ reactor}$ was increased. LDH activity was generally low and a marked increase only could be seen as lactate yield became dominant at higher glucose concentrations. PDC activity was not influenced as LDH. Ten fold increase in $C_{G,\ reactor}$, from 0.03-0.05 to 0.4-0.6 g l^{-1} increased LDH activity 7-8 fold, while PDC activity increased only 2.5 fold. PDC showed a substantial activity at different glucose availability. A similar situation was shown in *S. cerevisiae*, whose PDC was present at high levels at all dilution rates in aerobic glucose limited chemostat culture (55). ADH activity showed a

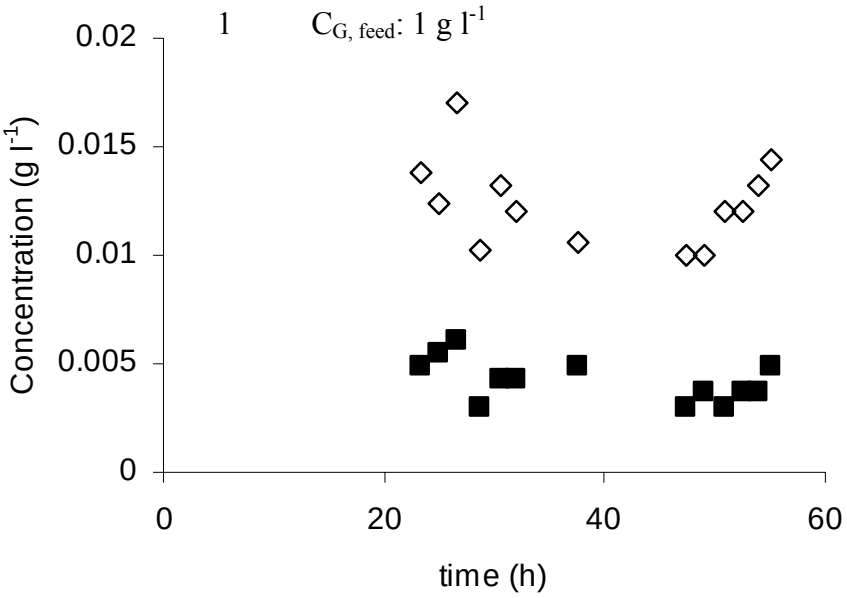
slight increase as $C_{G, reactor}$ was increased from 0.03-0.05 to 0.2-0.3 g l^{-1}, but remained at the same level as $C_{G, reactor}$ was increased to 0.5-0.6 g l^{-1}. Enzymatic activities were measured both in the first and the second day, in order to check for a possible pseudo steady state in the continuous cultures. Generally, the values were not different more than 16%, except the LDH activities observed at low glucose concentrations. At those cultures LDH activities were so low that they could be hardly measured. LDH and PDC activities were measured at similar pyruvate and NADH concentrations. Therefore, activity of the one of the enzymes may interfere with the assay of the other. Since PDC was more active, it may have interfered with the LDH assay. One solution to this problem is the incorporation of pyrazole, an ADH inhibitor, to LDH assay. However, this did not work, most probably due to high activity of ADH in *R. oryzae*. Another approach was to repeat the LDH assays by adding a LDH inhibitor, oxamate. In some of the samples, oxamate did not inhibit the activity completely, which was an evidence for the interference of PDC to LDH assays. Consequently, LDH activities reported in Table 3.3 could be more than the actual activities.

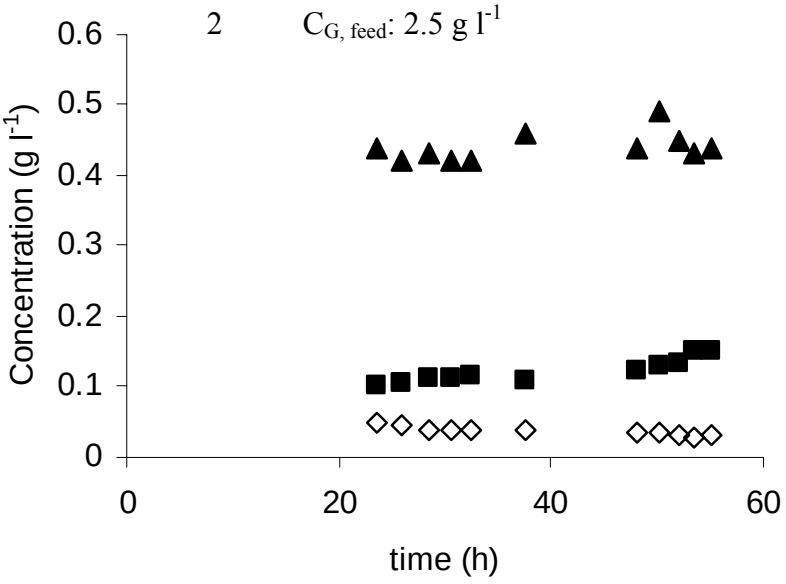
Dominance of ethanol production at low glucose levels can not be explained by the kinetics of the LDH and PDC enzymes of *R. oryzae.* Affinity of LDH for pyruvate is an order of magnitude lower than the affinity of PDC. If kinetics of the enzyme had determined the flux, lactate would have been produced at low glucose concentrations and ethanol production would have started or become dominant at higher glucose levels. Rather, levels (activities) of the enzymes may have determined the fate of the pyruvate. At low glucose concentrations LDH level was low and PDC was considerably high, therefore ethanol was produced more than lactate. When substantial LDH activity was observed, lactate production was high.

It was expected to minimize the production of fermentation end products (lactate and ethanol) and maximize the biomass production by keeping the concentration of the carbon source (glucose) low in the culture medium. Respiratory dissimilation of pyruvate may have been obtained under aerobic conditions at low glucose feeding rates. Reactor glucose concentration ($C_{G, reactor}$) was kept as low as

possible in order to avoid glucose repression on the respiratory enzymes and an overflow at the pyruvate level. All pyruvate was expected to be channeled to respiratory system and no pyruvate would be available for LDH and PDC. However, even at low concentrations of glucose (0.03-0.05 g l^{-1}) lactate and ethanol was observed. Ethanol production was unexpectedly high (0.17 g g glucose^{-1}) and was not influenced considerably as $C_{G,\ reactor}$ changed. Lactate yield, on the other hand, was very low at low $C_{G,\ reactor}$, however it was influenced greatly by glucose concentration. LDH and PDC specific activities showed similar trends with lactate and ethanol yields, respectively. LDH needed high glucose concentrations for high activity, which was reflected in lactate yields. Pritchard (57) showed that LDH activity was very low in batch *R. oryzae* cultures with 2.5-5 g l^{-1} initial glucose concentration. Increasing the glucose concentration to 10 and 20 g l^{-1} increased the LDH activity 4 and 9 folds, respectively. Our findings supported his findings, i.e. high level of glucose was needed for high LDH activity, hence lactic acid production.

As discussed at the beginning of the section, *R. oryzae* is difficult to cultivate in the bioreactor, since it attaches the inner elements of the reactor, which hinders the control of the process. Mycelia often clog the sparger, so that aeration is blocked. Even in well aerated cultures, the O_2 transfer, as well as other nutrients transfer, may be limited into the big mycelial mass formed around the elements of the reactor. Entrapment of the fungus inside Ca-alginate beads prevented these. Beads formed by the immobilization procedure described in Materials and Methods section had a diameter of 2.0-2.3 mm. Beads enlarge as the mycelium grows in them. Some free mycelia attached to impeller were observed. There were transport limitations for oxygen and nutrients to inner parts of the beads. That could be the reason for the ethanol production (recall that anaerobic conditions increase ethanol production).





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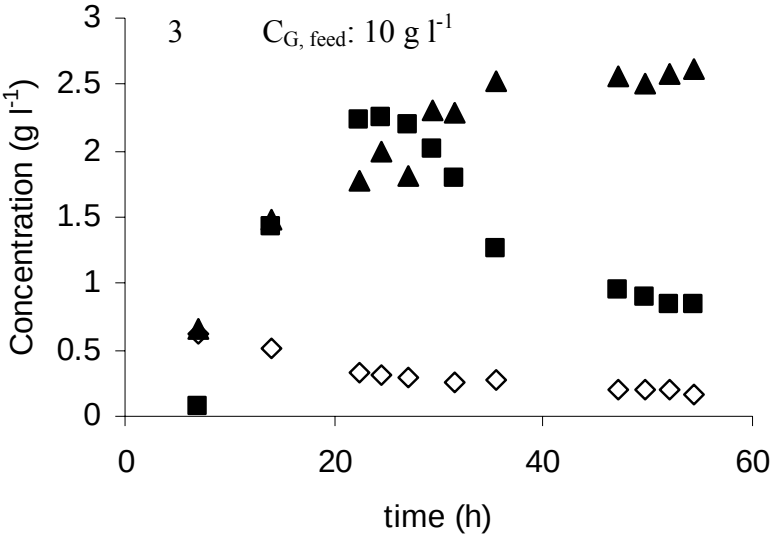

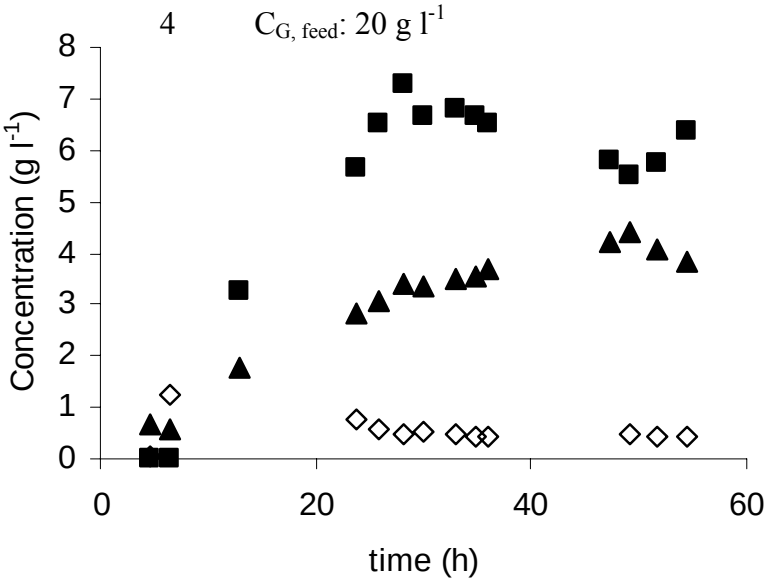

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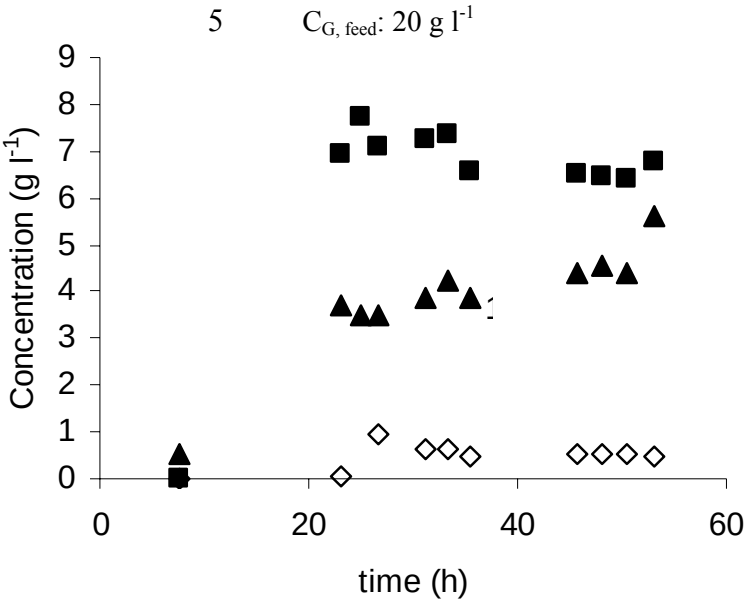

Figure 3.12. Fermentation kinetics in continuous cultures with cell retention, run number 1-5, which had different feed glucose concentrations. Feeding start time is designated as time 0. glucose (◊),lactate (■),ethanol (▲).

Table 3.2. Lactate, ethanol and biomass levels and yields in continuous alginate immobilized *R. oryzae* cultures

Run Number	**$C_{G,\ feed}$ (g l^{-1})**	**G_{fed} (g)**	**$C_{G,reactor}$ (g)**	**L (g)**	**Y_{SL} (g g^{-1})**	**E (g)**	**Y_{SE}(g g^{-1})**	**X (g)**	**Y_{SX}(g g^{-1})**
1	1	6.84	0.010-0.014	0.03	0.004	n.d.	n.d.	1.12	0.175
2	2.5	18.3	0.03-0.05	0.85	0.05	3.1	0.17	2.7	0.15
3	10	88	0.2-0.3	12.2	0.14	20.3	0.24	8.2	0.10
4	20	163	0.5-0.6	49.5	0.33	29.5	0.20	7.7	0.05
5	20	160	0.5-0.6	44.4	0.28	25.4	0.16	7.5	0.05

L and E are the total amounts of lactate and ethanol drawn out in the effluent stream, respectively.
G_{fed} is the total amount of glucose added to the culture.

Table 3.3. Specific activities of pyruvate branch point enzymes in continuous alginate immobilized *R. oryzae* cultures.

Run Number	$C_{G,reactor}$ (g l^{-1})	LDH (U mg protein^{-1})		PDC (U mg protein^{-1})		ADH (U mg protein^{-1})	
		Day1	Day2	Day1	Day2	Day1	Day2
1	0.010-0.014	0.027	0.046	0.25	0.29	11.6	12.9
2	0.03-0.05	0.087	0.062	0.84	0.94	16.0	16.7
3	0.2-0.3	n.d.	0.13	1.4	1.3	21.5	22.3
4	0.4-0.6	0.52	0.48	2.0	2.2	21.5	22.3
5	0.5-0.6	0.62	0.52	2.2	2.1	24.5	20.6

3.2.3. Glucose Pulse

Alginate immobilized *R. oryzae* was pregrown in shake flask and then cultivated semi-continuously in bioreactor by feeding low amount ($C_{G,\ feed}$=1 g l^{-1}) of glucose for 40 h. Feed glucose concentration was 1 g l^{-1}. Twenty grams glucose was pulsed and lactate and ethanol levels and enzymatic activities were followed for 13.5 h. Time of glucose pulse was taken as zero. Approximately 17.5 g glucose was utilized and 6.2 g lactate and 3.7 g ethanol was produced (Figure 3.12). Generally, glucose utilization and lactate production rates were increased in time, while ethanol production rate was almost the same in the same period. Ethanol concentration increased almost linearly after the glucose pulse. Unlike ethanol, lactate production was slow at the beginning and increased in time. Lactate production became dominant 7-8 h after the glucose pulse.

At zero time LDH, PDC and ADH activities were 0.071, 0.7 and 16.3 U mg $protein^{-1}$, respectively (Figure 3.13). Activities increased almost linearly after the glucose pulse. PDC activity increased slightly faster than LDH activity (0.08 and 0.10 U mg $protein^{-1}$ h^{-1}, respectively). In 13.5 h, LDH, PDC and ADH activities reached 1.2, 2.1 and 30 U mg $protein^{-1}$, respectively. Consequently, activities of LDH, PDC and ADH increased to 16, 3 and 1.8 fold. In agreement with the results presented in previous section, PDC and ADH showed substantial *in vitro* activity before the glucose pulse, which support immediate production of ethanol after the pulse. Since LDH activity was very low at the time of glucose pulse, it took several hours for lactate production to be dominant, although LDH activity increased parallel to the PDC activity.

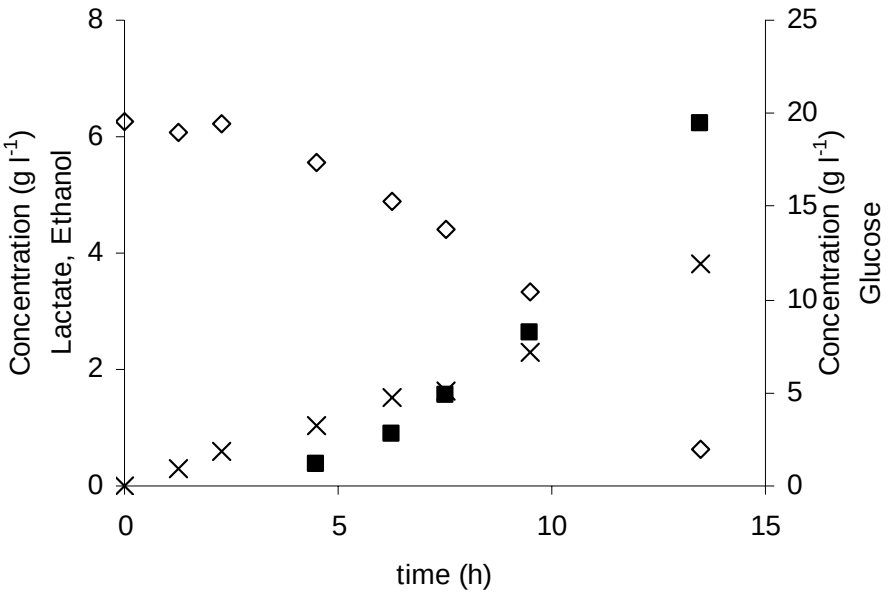

Figure 3.13. Time course of fermentation after the glucose pulse. Glucose (◊), lactate (■), ethanol (x)
Time of glucose pulse is designated as time zero

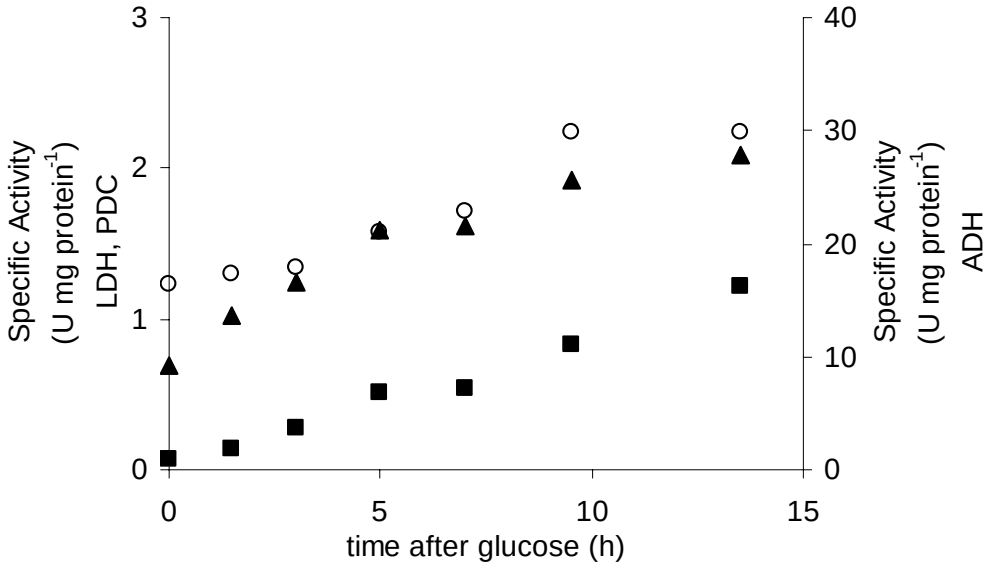

Figure 3.14. Time course enzymatic activities after the glucose pulse. LDH (■),PDC (▲), ADH (○)
Time of glucose pulse is designated as time zero

3.2.4. Fed-batch Culture

In an effort to minimize the glucose concentration in order to maximize biomass yield, *R. oryzae* was cultivated in fed-batch mode. Alginate immobilized cells were pre-grown in shake flask and transferred to bioreactor to start glucose feeding. Feed contained 20 g l^{-1} glucose and mineral salts at 1X concentration. Feeding rate was 3.4 ml h^{-1} at time zero and increased to 17.1 ml h^{-1} linearly in 72 h. Total of 740 ml feed, which contained 14.8 g glucose was added. Dry cell weight increased from 0.76 to 4.52 g, so that Y_{SX} was calculated as 0.25 g DCW per g glucose utilized. Glucose was not detected and lactate and ethanol concentrations were very low. After 72 h only 1 g ethanol and less than 0.1 g lactate was measured. Thus, ethanol yield was 0.07 and lactate yield was 0.007 g g glucose $^{-1}$.

Lactate and ethanol yields could be kept quite low and biomass yield was high; however biomass yield, like in the other experiments in this dissertation, was lower than expected. In glucose limited aerobic cultivation *S. cerevisiae* Y_{SX} of 0.5 can be achieved in aerobic glucose limited chemostat cultures.

CHAPTER 4

CONCLUSION

Rhizopus oryzae ATCC 9363 has two fermentative pathways, lactic acid and ethanol producing pathways, both of which utilize pyruvate. This work gave us some clues about the regulation of the pyruvate branch point. Flux through the fermentative pathways was shown to be influenced by environmental conditions. PDC, being active at all external glucose concentration, should be the first target for knock-out studies in an effort to increase lactate yield. On the other hand, considerable ethanol production and ability to ferment starch make this fungus a potential organism for bioethanol production. LDH levels were influenced by the environmental conditions greatly. Therefore, overexpression of ldh gene(s) may increase lactate production. Clumped growth form was always avoided in lactate fermentation by *R. oryzae* due to the mass transfer difficulty. However, in this study comparable lactate yield could be obtained with clumped morphology. This shows that morphology of this fungus and its effect on physiology needs more in depth studies.

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APPENDIX A

DEFINITIONS OF YIELD COEFFICIENTS

Yield on substrate:

Lactic acid (lactate) yield: $$Y_{SL} = \frac{\text{lactic acid produced (g l}^{-1})}{\text{substrate (glucose) consumed (g l}^{-1})}$$

Ethanol yield: $$Y_{SE} = \frac{\text{ethanol produced (g l}^{-1})}{\text{substrate (glucose) consumed (g l}^{-1})}$$

Biomass yield: $$Y_{SX} = \frac{\text{biomass produced (g l}^{-1})}{\text{substrate (glucose) consumed (g l}^{-1})}$$

Yield on biomass:

Lactic acid (lactate) yield: $$Y_{XL} = \frac{\text{lactic acid produced (g l}^{-1})}{\text{biomass produced (g l}^{-1})}$$

Ethanol yield: $$Y_{XE} = \frac{\text{ethanol produced (g l}^{-1})}{\text{biomass produced (g l}^{-1})}$$

APPENDIX B

SAMPLE HPLC CHROMATOGRAM

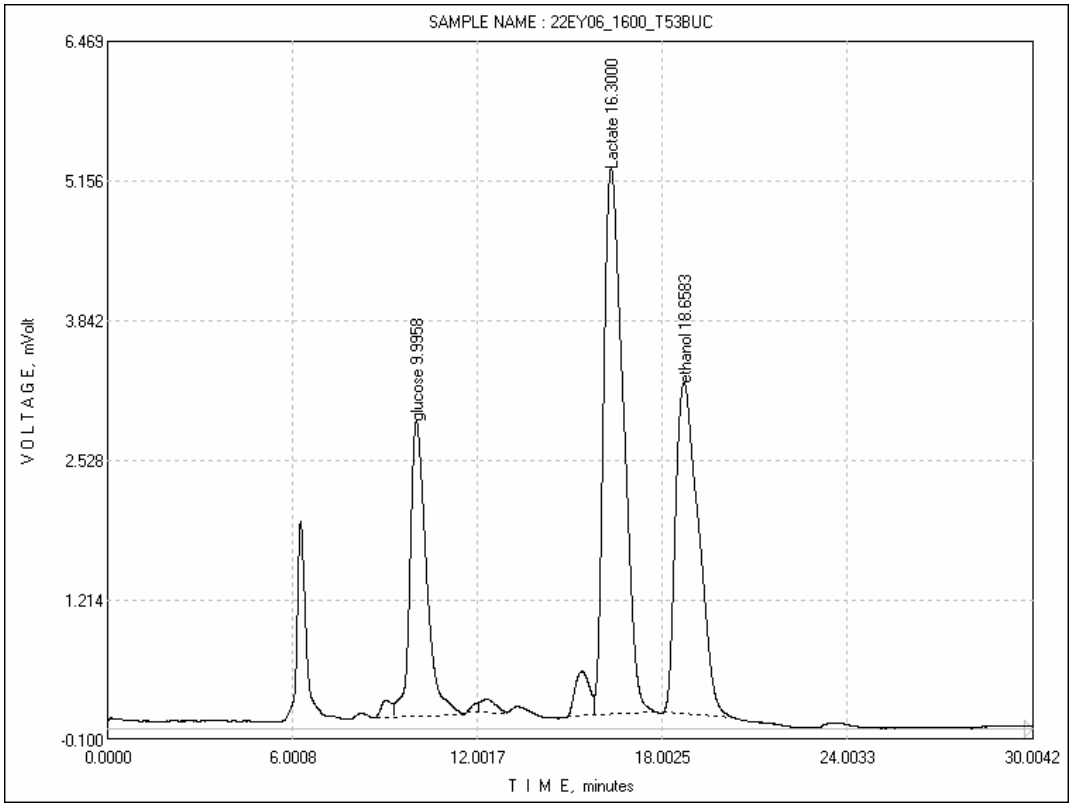

Figure A.1. A sample HPLC chromatogram showing the glucose , lactate and ethanol peaks

APPENDIX C

CARBON MASS BALANCE CALCULATION

Maximum biomass yield in *S. cerevisiae* culture is around 0.5 g DCW g glucose^{-1}. Therefore, it is assumed that 2 g glucose is consumed for the production of 1 g of *R. oryzae* biomass (DCW). The other 1 g is lost as CO_2 and H_2O in biomass forming pathways.

One mole of carbon dioxide (CO_2) produced for production of 1 mole of ethanol. Therefore 1.96 g glucose is consumed for the production of 1 g ethanol (Mw of ethanol = 46 g mole^{-1}, Mw of CO_2 = 44 g mole^{-1}).

Mass balance:
Input: Initial Glucose (G_o)
Output: Final glucose (G_f), Lactate (L) , Ethanol (E), Biomass (X)

$$G_o\ (g) \longrightarrow G_f\ (g) + L\ (g) + 1.96\ E\ (g) + 2\ X\ (g)$$

Sample mass balance:
Data from a shake flask culture
Initial glucose concentration ($Glucose_i$) = 100 g l^{-1}
Final glucose concentration ($Glucose_f$) = 0 g l^{-1}
Lactate concentration = 78.4 g l^{-1}
Ethanol concentration = 7.7 g l^{-1}
Biomass concentration = 2.3 g DCW l^{-1}
Input = 100 g l^{-1}
Output = 0 + 78.4 + 1.96 x 7.7 + 2 x 2.3 = 93.5 g l^{-1}
Balance shows that 93.5 % of the initial glucose was accounted for extracellular metabolites and biomass.

APPENDIX D

KINETIC ANALYSIS OF PRODUCT FORMATION IN TERMS OF ENZYMATIC ACTIVITIES

Lactate dehydrogenase follows normal Michaelis Menten kinetics with respect to pyruvate (57), while pyruvate decarboxylase obeys the Hill equation (1).

Hill equation: $V = \frac{V_{max} S^n}{K_m + S^n}$

Michaelis-Menten equation: $V = \frac{V_{max} S}{K_m + S}$

V: rate of the enzymatic reaction (mmol l^{-1} min^{-1})

V_{max}: maximum rate of the reaction

S: substrate concentration (mmol l^{-1})

K_m: substrate concentration when $V=V_{max}/2$

n: Hill coefficient (n=2 for PDC)

To calculate the intracellular rate of lactate and ethanol production, mycelial volume of 1.2 µl per mg of dry cell was assumed (64; 21). Specific activities measured in the cell extracts were taken as the maximum rates (V_{max}). Cytosolic volume is assumed to be equal to 2.4 µl mg $protein^{-1}$. Therefore measured enzymatic activities (U mg $protein^{-1}$) were multiplied by 417 to convert the unit to mmol l $cytosol^{-1}$ min^{-1}.

K_m values for pyruvate of LDH and PDC are taken from the literature as 0.6 and 4.2 mM (average of the K_m values of the two isoenzymes, 3.9 and 4.5 mM (1)), respectively.

Case 1: Lactate producing batch culture.

Dry cell weight at the end of the culture was 0.1125 g. Therefore, cytosolic volume was calculated as 0.135 ml. Since the total culture volume is 50 ml, rate should be 50/0.135 times higher in the cytosol. Therefore, the rate of product formation should be approximately 400 fold of the rate in the culture media.

Rate of product formations measured in the culture:

$$\frac{d(lactate)}{dt} = 0.52 \text{ mmol } l^{-1} \text{ min}^{-1}$$

$$\frac{d(ethanol)}{dt} = 0.13 \text{ mmol } l^{-1} \text{ min}^{-1}$$

Rate of product formations in the cytosol were calculated as:

$$\frac{d(lactate)}{dt} = 208 \text{ mmol } l^{-1} \text{ min}^{-1}$$

$$\frac{d(ethanol)}{dt} = 52 \text{ mmol } l^{-1} \text{ min}^{-1}$$

V_{max} values measured in cell extracts are:
LDH: 2.4 U mg protein^{-1} (μmol min^{-1} mg protein^{-1})
PDC: 1.3 U mg protein^{-1} (μmol min^{-1} mg protein^{-1})

V_{max} values calculated for the activities in the cytosol
LDH: 1000.8 mmole min^{-1} l cytosol^{-1}
PDC: 542.1 mmole min^{-1} l cytosol^{-1}

Case 2: Ethanol producing batch culture.

Dry cell weight at the end of the culture was 0.23. Therefore, cytosolic volume was calculated as 0.28 ml cytosol. Since the total culture volume is 50 ml, rate

should be 50/0.28 times higher in the cytosol. Therefore, the rate of product formation should be approximately 200 fold of the rate in the culture media.

Rate of product formations measured in the culture:

$$\frac{d(lactate)}{dt} = 0.19 \text{ mmol } l^{-1} \text{ min}^{-1}$$

$$\frac{d(ethanol)}{dt} = 0.58 \text{ mmol } l^{-1} \text{ min}^{-1}$$

Rate of product formations in the cytosol were calculated as:

$$\frac{d(lactate)}{dt} = 38 \text{ mmol } l^{-1} \text{ min}^{-1}$$

$$\frac{d(ethanol)}{dt} = 116 \text{ mmol } l^{-1} \text{ min}^{-1}$$

V_{max} values measured in cell extracts are:
LDH: 0.3 U mg protein^{-1} (μmol min^{-1} mg protein^{-1})
PDC: 1.8 U mg protein^{-1} (μmol min^{-1} mg protein^{-1})

V_{max} values calculated for the activities in the cytosol
LDH: 125.1 mmole min^{-1} l cytosol^{-1}
PDC: 750.6 mmole min^{-1} l cytosol^{-1}

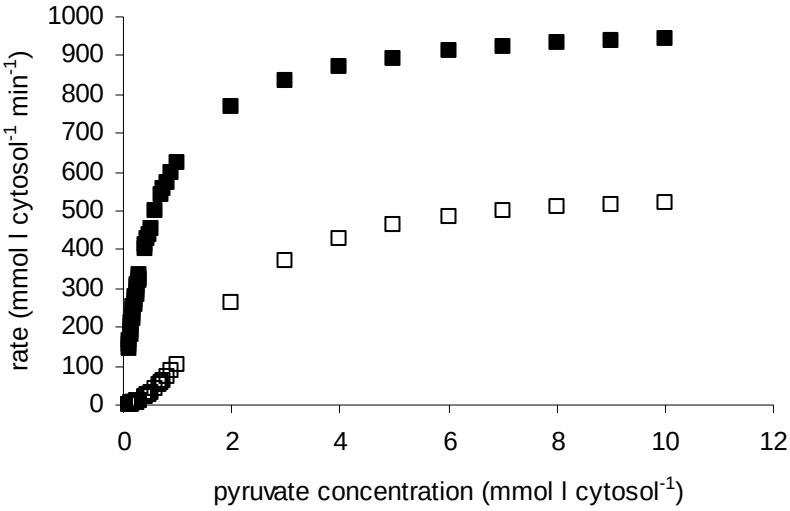

Figure A.2. Dependence of rate of LDH (closed squares) and PDC (open squares) catalyzed reactions on pyruvate concentration according to the Michealis-Menten equation in lactate producing batch culture

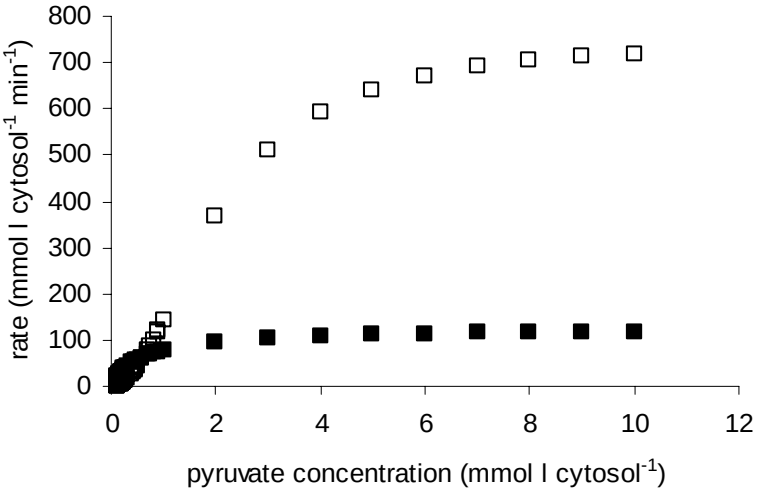

Figure A.3. Dependence of rate of LDH (closed squares) and PDC (open squares) catalyzed reactions on pyruvate concentration according to the Hill equation in ethanol producing batch culture

In order to calculate K_m and V_{max} values for a given pyruvate concentration, following equations derived from the reciprocals of the Michaelis-Menten and Hill equations were used:

Michaelis-Menten equation used for LDH:

$$\frac{1}{V} = \frac{1}{V_{\max}}\left(\frac{K_m}{S} + 1\right), \quad V_{\max} = V\left(\frac{K_m}{S} + 1\right), \quad K_m = V_{\max} S\left(\frac{1}{V} - \frac{1}{V_{\max}}\right)$$

Hill equation used for PDC:

$$\frac{1}{V} = \frac{1}{V_{\max}}\left(\frac{K_m}{S^2} + 1\right), \quad V_{\max} = V\left(\frac{K_m}{S^2} + 1\right), \quad K_m = V_{\max} S^2\left(\frac{1}{V} - \frac{1}{V_{\max}}\right)$$

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PUBLICATIONS

1. Büyükkileci A.O., Hamamcı H., Yücel M., "Lactate and Ethanol Production by *Rhizopus oryzae* and activities of related pyruvate branch point enzymes, Journal of Bioscience and Bioengineering, 5(12), 225-228 (2006)

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HOBBIES

Music, Sports