AROMATIC AMINO ACID SYNTHESIS PERFORMANCE OF *Bacillus acidocaldarius*

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ABSTRACT

AROMATIC AMINO ACID SYNTHESIS PERFORMANCE OF *Bacillus acidocaldarius*

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In this study, the effects of bioprocess operation parameters on aromatic amino acid synthesis performance of *Bacillus acidocaldarius* were investigated. Firstly, in laboratory scale shake-bioreactors, a defined medium was designed in terms of its carbon and nitrogen sources, to achieve the highest cell concentration. Thereafter, the effects of bioprocess operation parameters, i.e., pH and temperature were investigated; and the optimum medium contained (kg m^{-3}): fructose, 8; $(NH_4)_2HPO_4$, 5; $CaCl_2$, 0.2; KH_2PO_4, 2; $NaH_2PO_4.2H_2O$, 7.318; Na_2HPO_4, 0.0438; $Mg(CH_3COO)_2.4H_2O$, 87×10^{-3} ; 1 , $MgSO_4.7H_2O$; 2×10^{-3}, $FeSO_4.7H_2O$; 2×10^{-3}, $ZnSO_4.7H_2O$; 15 $\times10^{-5}$, $MnSO_4.H_2O$; 2×10^{-5}, $CuSO_4.5H_2O$ with pH_0 =5, T=55°C, N=175 min^{-1}. In this

medium, the bacteria produced L-tryptophan at the highest concentration of 0.204 kg m^{-3} and L-phenylalanine at a maximum concentration of 0.0106 kg m^{-3} with no L-tyrosine production. Finally the fermentation and oxygen transfer characteristics of the bioprocess were investigated in 3.0 dm^3 pilot scale bioreactors. The effects of oxygen transfer were investigated at four different conditions at the parameters air inlet rates of Q_O/V_R =0.2, and 0.5 vvm, and agitation rates of N= 250, 500, 750 min^{-1}. The effect of pH was investigated at pH=5 uncontrolled and controlled operations. The variations in cell, fructose, amino acid and organic acid concentrations with the cultivation time; and using the dynamic method, the oxygen uptake rate and the liquid phase mass transfer coefficient values throughout the growth phase of the bioprocess; the yield and maintenance coefficients were determined. The aromatic amino acids produced at the highest and the least amount and frequency were L-tryptophan and L-tyrosine, respectively. The highest L-tryptophan production, 0.32 kg m^{-3} in 17 hour was at 0.2 vvm and 500 min^{-1}. Among all operations, the highest L-tryptophan was produced at the lowest oxygen transfer condition. Controlled-pH conditions produced more L-tryptophan.

Keywords: Aromatic amino acid, production, *Bacillus*, medium design, bioprocess operation parameters

ÖZ

BACILLUS ACIDOCALDARIUS'UN AROMATİK AMİNO ASİT ÜRETİM PERFORMANSI

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Bu çalışmada, biyoproses tasarım parametrelerinin, *Bacillus acidocaldarius'*un aromatik amino asit üretim performansına etkileri incelenmiştir.İlk olarak, laboratuvar ölçekli biyoreaktörlerde, en yüksek hücre derişimi için karbon ve azot kaynaklarının etkileri araştırılarak tanımlanmış bir ortam tasarlanmıştır.Sonra, biyoproses işletim parametrelerinden pH ve sıcaklığın etkileri incelenmiştir. En uygun ortam ; pH_0 =5, T=55°C ve N=175 min^{-1} koşullarında; (kg m^{-3}): fruktoz, 8; $(NH_4)_2HPO_4$, 5; $CaCl_2$, 0.2; KH_2PO_4, 2; $NaH_2PO_4.2H_2O$, 7.318; Na_2HPO_4, 0.0438; $Mg(CH_3COO)_2.4H_2O$, 87×10^{-3} ; 1 , $MgSO_4.7H_2O$; 2×10^{-3}, $FeSO_4.7H_2O$; 2×10^{-3}, $ZnSO_4.7H_2O$; 15 $\times10^{-5}$, $MnSO_4.H_2O$; 2×10^{-5}, $CuSO_4.5H_2O$ içermektedir. Bu ortamda, bakteri en yüksek 0.204 kg m^{-3} derişiminde L-triptofan ve en yüksek 0.0106 kg m^{-3} derişiminde L-fenilalanin üretmiştir, fakat L-tirozin üretmemiştir. Son olarak,

fermantasyon ve oksijen aktarımı karakteristikleri, 3.0 dm^3 hacimli pilot ölçekli biyoreaktörde incelenmiştir. Pilot ölçekli biyoreaktörde, oksijen aktarım etkileri hava giriş hızının Qo/V_R =0.2 ve 0.5 vvm ve karıştırma hızının N=250, 500, 750 min^{-1} olduğu 4 farklı koşulda incelenmiştir. pH'ın etkisi başlangıç pH'nın 5 olduğu kontrollu ve kontrolsuz pH işletim koşullarında incelenmiştir. Hücre, fruktoz, amino asit ve organik asit konsantrasyonlarının kalma süresi ile değişimi; dinamik yöntem kullanılarak, biyoprosesin büyüme evresi süresince, oksijen tüketim hızı ve sıvı faz kütle aktarım katsayısı; verim ve yaşam katsayıları belirlenmiştir. En çok ve en az miktarda ve sıklıkta üretilen aromatik aminoasitler,sırasıyla, L-triptofan ve L-tirozindir. En yüksek L-triptofan üretimi, ilk 17 saatte 0.32 kg m^{-3} , 0.2 vvm and 500 min^{-1} koşullarında elde edilmiştir. Tüm koşullardaki en yüksek L-triptofan üretiminin, oksijen aktarım koşullarının en düşük olduğu şartta elde edildiği görülmüştür. pH-kontrollü koşulların, pH-kontrolsüz koşullardan daha yüksek miktarlarda L-triptofan ürettiği görülmüştür.

Anahtar Kelimeler: Aromatik amino asit, üretim, *Bacillus*, ortam tasarımı, biyoproses işletim parametreleri

To My Family

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

NOMENCLATURE

a	Specific Interfacial Surface Area, $m^2\ m^{-3}$
C_{OA}	Organic acid concentration, kg m^{-3}
C_{AA}	Amino acid concentration, kg m^{-3}
$\Sigma\ C_{OA}$	Total Organic acid concentration, kg m^{-3}
$\Sigma\ C_{AA}$	Total Amino acid concentration, kg m^{-3}
C_{DO}	Dissolved oxygen concentration, mol m^{-3}; kg m^{-3}
C_O^*	Oxygen saturation concentration, mol m^{-3}; kg m^{-3}
C_F	Fructose concentration, kg m^{-3}
C_F^0	Initial fructose concentration, kg m^{-3}
C_G	Glucose concentration, kg m^{-3}
C_G^0	Initial glucose concentration, kg m^{-3}
C_P	Product concentration, kg m^{-3}
C_{TRP}	L-Tryptophan concentration; kg m^{-3}
C_{PHE}	L-Phenylalanine concentration; kg m^{-3}
C_{TYR}	L-Tyrosine concentration; kg m^{-3}
C_X	Cell concentration, kg dry cell m^{-3}
Da	Damköhler number (=OD / OTR_{max} ; Maximum possible oxygen utilization rate per maximum mass transfer rate)
D	Diameter of the bioreactor, m
d_b	Diameter of bubble, m
d_O	Diameter of the orifice, m
D_I	Diameter of the impeller, m
E	Enhancement factor (=K_La / K_La_o); mass transfer coefficient with chemical reaction per physical mass transfer coefficient
HOT- pH_{UC}	Qo/V_R=0.5 vvm+N=750 min^{-1} +uncontrolled pH experiment
k_L	Liquid phase mass transfer coefficient
K_La	Overall volumetric liquid phase mass transfer coefficient; s^{-1}
LimOT-pH_C	Qo/V_R=0.2 vvm+ N=250 min^{-1} +controlled pH experiment
LOT-pH_C	Qo/V_R=0.5 vvm+ N=250 min^{-1} +controlled pH experiment
LOT-pH_{UC}	Qo/V_R=0.5 vvm+ N=250 min^{-1} +uncontrolled pH experiment
MOT-pH_C	Qo/V_R=0.5 vvm+ N=500 min^{-1} +controlled pH experiment

MOT-pH_{UC}	Qo/V_R=0.5 vvm+N=500 min^{-1} +uncontrolled pH experiment
N	Agitation rate, min^{-1}
N_P	Power number
ni	Number of Impellers
no	Number of Orifices
m_0	Rate of oxygen consumption for maintenance, g oxygen g^{-1} dry cell weight h^{-1}
m_S	Maintenance coefficients for substrate, g substrate g^{-1} dry cell weight h^{-1}
P	Power of the bioreactor
pH_0	Initial pH
pH_C	Controlled pH
Qo	Volumetric air feed rate, m^3 min^{-1}
q_o	Specific oxygen uptake rate, kg kg $^{-1}$ DW h^{-1}
q_s	Specific substrate consumption rate, kg kg $^{-1}$ DW h^{-1}
q_P	Specific product formation rate, kg kg $^{-1}$ DW h^{-1}
r_0	Oxygen uptake rate, mol m^{-3} s^{-1}; kg m^{-3} h^{-1}
r_S	Substrate consumption rate, kg m^{-3} h^{-1}
r_P	Product formation rate, kg m^{-3} h^{-1}
r_X	Rate of cell growth, kg m^{-3} h^{-1}
T	Bioreaction medium temperature, °C
t	Bioreactor cultivation time, h
V	Volume of the bioreactor, m^3
V_R	Volume of the bioreaction medium, m^3
$Y_{X/S}$	Instantaneous Yield of cell on substrate, g g^{-1}
$Y_{X/O}$	Instantaneous Yield of cell on oxygen, g g^{-1}
$Y_{S/O}$	Instantaneous Yield of substrate on oxygen, g g^{-1}
$Y'_{/X}$	Observed Overall Yield of product on cell, g g^{-1}
$Y'_{/S}$	Observed Overall Yield of product on substrate, g g^{-1}
$Y'_{X/S}$	Observed Overall Yield of cell on substrate, g g^{-1}
$Y'_{P/X}$	Observed Overall Yield of product on cell, g g^{-1}
$Y'_{P/S}$	Observed Overall Yield of product on substrate, g g^{-1}
$\overline{Y}$	True Overall Yield
u_b	Bubble velocity, m s^{-1}

Greek Letters

α	Leudeking Piret constant, g g^{-1} h^{-1}

β	Leudeking Piret costant, g g^{-1}
ε	Gas Hold up
σ	Surface Tension, N m^{-1}
η	Effectiveness factor (=OUR/OD; the oxygen uptake rate per maximum possible oxygen utilization rate)
η*	Viscosity, kg m^{-1} s^{-1}
μ	Specific cell growth rate, h^{-1}
μ_{max}	Maximum specific cell growth rate, h^{-1}
λ	Wavelength, nm

ABBREVIATIONS

Ac	Acetic acid
Arg	Arginine
Ala	Alanine
Asn	Asparagine
Asp	Aspartic acid
α-KG	α-ketoglutarate
But	Butyric acid
Cit	Citrate
Cys	Cysteine
DNS	Dinitrosalicilyc acid
DO	Dissolved oxygen
D_{O2}	Diffusivity of oxygen, m^2 s^{-1}
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen
Gln	Glutamine
Glu	Glutamic acid
Gluc	Gluconic acid
His	Histidine
Iso	Isoleucine
Lac	Lactic acid
Leu	Leucine
NRLL	Northern Regional Research Center
Phe	Phenylalanine
Pro	Proline

Pyr	Pyruvate
Re	Reynolds number (=duρ/η*)
OD	Oxygen demand (=μ_{max} C_X / $Y_{X/O}$; mol m^{-3} s^{-1})
OUR	Oxygen uptake rate, mol m^{-3} s^{-1}
OTR	Oxygen transfer rate, mol m^{-3} s^{-1}
OTR_{max}	Maximum possible mass transfer rate (=$K_L a C_O$*; mol m^{-3} s^{-1})
Sc	Schmidt Number (=η*/(ρD_{O2}))
Ser	Serine
Sh	Sherwood Number (=$k_L$db/D_{O2})
Suc	Succinic acid
Thr	Threonine
Tyr	Tyrosine
Trp	Tryptophan
Val	Valine

CHAPTER 1

INTRODUCTION

Amino acids are the basic building blocks of proteins . Most amino acids are alpha amino acids which have a common backbone of an organic carboxyl (-COOH) group, an amino ($-NH_2$) group and a hydrogen atom (H) attached to a saturated alpha (α) carbon atom. What makes the difference between the amino acids is the R group attached to the alpha carbon. The amino acids are differentiated and named according to the R group attached to the alpha carbon. (Bailey and Ollis, 2^{nd} edition, 1986).

Aromatic amino acids are the ones containing aromatic ring as their side chain. Phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) are the aromatic amino acids. The phenylalanine R group contains a benzene ring, tyrosine contains a phenol group and the tryptophan R group contains the heterocyclic structure, indole (T. Devlin, 1997,4^{th} edition). Trp and Phe are essential amino acids while Tyr is nonessential. Tyr is the precursor of some important neurotransmitters like epinephrine, norepineprine and dopamine (Bohinski, 5^{th} edition,1987). Trp, an essential amino acid, is the precursor to serotonin. Phe is important for the development and maintenance of humans.

Aromatic amino acids have common reaction steps in their biosynthesis with the microorganisms.

Chemical synthesis of the amino acids has the disadvantage of being expensive and particularly inefficient because it makes a mixture of both L- and D-isomers. Microbial production yields only biologically active L-isomer and therefore seems to be a more promising way of amino acid production (Torres et al., 2000).

As overproduction of amino acids in the wild-type microbial cells is hard because of the complex regulations of feedback inhibition and repressions, like other amino acids, aromatic amino acids are generally produced by genetically modified microorganisms. Besides chemical and enzymatic synthesis, Trp is produced by genetically modified strains of *E.coli* and *Corynebacterium glutamicum*, *Brevibacterium flavum* (Devlin, 4th edition, 1997 ; Moses and Cape, 2nd edition, 1994) and *Claviceps purpurea, Hansenula anomala, Bacillus subtilis* (Atkinson & Mavituna, 1991). It is used in medicine , especially in the design of antidepressants and sleep-inducers. Its production is 250 ton/year with a price range of 74-147 $/kg. Phe is produced from *Brevibacterium lactofermentum* and Tyr is produced from *Corynebacterium glutamicum* and *B. Lactofermentum Glutamicum* (Moses and Cape, 2nd edition, 1994). Being an essential amino acid, Phe is used in food supplements. Its production is 3000 ton /year; with a price range of 59-88 $/kg. Phe can also be synthesised via chemical and enzymatic ways. Tyr ,used in food supplements and medicine, can also be produced by extraction besides fermentation. Its production is 60 ton/year, with a price range of 74-110 $/kg (Kirk and Othmer, 1994).

In the literature there are many studies on genetic and medium design for overproduction of the aromatic amino acids. Hagino and Nakayama (1973) were able to select organisms that could accumulate 17.6 kg m^{-3} tyrosine in 96 h on a cane molasses medium; by combining auxotrophy and analog resistance.In molasses media, Hagino and Nakayama (1974) achieved 9.5 kg m^{-3} Phe in 96 h with mutants of *C. Glutamicum*. Kawasaki et al. (1996) reported L-tryptophan production by a pyruvic acid-producing *E. coli* carrying the tryptophanase structural gene from *Enterobactera aerogenes* downstream

of the tac promoter. Dodge and Gernstner (2002) , by using an engineered strained of *E.coli*, developed a fed-batch fermentation process and increased the yield on glucose to 0.176 producing 42.3 kg m^{-3} Trp. However in the literature there is no systematic investigation on the effects of bioprocess operation parameters on aromatic amino acid production. Also in the literature there is no work on aromatic amino acid production by the acidophilic-thermophilic bacteria, *Bacillus acidocaldarius*.

In this study, the effects of bioprocess design parameters on aromatic amino acid synthesis capacity of *Bacillus acidocaldarius* were investigated. In this context, a defined medium was designed in terms of its carbon and nitrogen sources, in order to achieve a highest cell concentration. Thereafter, by using the designed medium, the effects of bioprocess operation parameters, i.e., pH and temperature, on biomass concentration were investigated in laboratory scale bioreactors. Finally, using the optimum bioprocess parameters obtained in the previous steps, effects of oxygen transfer and pH strategy on aromatic amino acid synthesis performance of *Bacillus acidocaldarius*, the fermentation and oxygen transfer characteristics of the bioprocess were investigated in pilot scale bioreactors.

CHAPTER 2

LITERATURE SURVEY

2.1 Amino acids

2.1.1 Properties of Amino acids

Amino acids are the basic building blocks of polypeptides, proteins and enzymes. They are very small biomolecules with an average molecular weight of 135 daltons. Although in nature there are three hundred different amino acids, all living organisms use only twenty of this number in the biosynthesis of proteins. Most amino acids are alpha amino acids which have a common backbone of an organic carboxyl (-COOH) group, an amino ($-NH_2$) group and a hydrogen atom (H) attached to a saturated alpha (α) carbon atom, whose general structure can be seen in Figure 2.1.

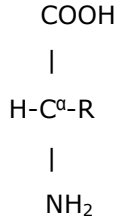
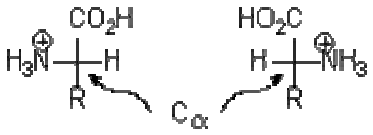


Figure 2.1 General Structure of an alpha amino acid

The amino acids are differentiated and named according to the R group attached to the alpha carbon. R represents a side chain specific to each amino acid. The R group can vary from a single hydrogen atom in glycine (the simplest amino acid) to a more complex structure such as a cyclic secondary amine structure in proline.

As the alpha carbon atom in amino acids, except glycine, is tetrahedrally attached to four different atoms, the alpha carbon is asymmetrical. This is the reason why amino acids are chiral or asymetric; thus optically active. Optical activity causes amino acids existing in 2 forms whose structures are non-super-imposable mirror images of each other: These isomeric forms are L- and D- form. This nomenclature arises from the fact that solutions of pure isomer will rotate plane polarized light either to the right (dextrorotary- or D-) or left (levorotary- or L-) . In Figure 2.2, L and D amino acids can be seen. In most proteins, only L-amino acid isomers are found while appearance of D-amino acids in nature is rare.

D-amino acids are only found in cell walls of some microorganisms and in some antibiotics. Thus this isomerism is very important as viable organisms can only utilize L (levatory) form ; not having the enzymes to convert D-amino acids into L-amino acids. (Bailey and Ollis, 2nd edition, 1986).



L form D form

Figure 2.2 Mirror Isomers

1. Neutral/Basic/Acidic

If the R group in the amino acid structure does not ionize, R group is neutral and carries no charge. When it can ionize, the R group is basic if it becomes a positively charged R group; or it is acidic if it becomes a negatively charged group.

2. Polar/Nonpolar R groups

A polar R group is hydrophilic (water-loving) and readily solvated by polar water molecules, by forming hydrogen bonding with water. A nonpolar R group is hydrophobic, not readily solvated by water molecules. Nonpolarity is because of the absence or minimal contribution of polarity and the presence of many nonpolar C-C and C-H bonds.

3. Human Nutrition Classification

Some amino acids, required for human diet, can not be produced from other substances. Thus they are essential amino acids. The deprivation of these amino acids can be disastrous. The essential amino acids are threonine, methionine, valine, leucine, isoleucine, phenylalanine, tryptophan, lysine, arginine and histidine. The other amino acids, which can be synthesized in human body, are called nonessential amino acids.

The acid (-COOH) and base ($-NH_2$) groups of amino acids can ionize in aqueous solution. The amino acid is positively charged (cation) at low pH and negatively charged (anion) at high pH.

The acidic group is neutral (-COOH) at low pH and negatively charged ($-COO^-$) at high pH. The basic group is neutral (NH_2) at high pH and positively charged (H_3N^+) at low pH. At an intermediate pH value, the amino acid acts as a dipolar ion or zwitterion, which has negatively and positively charged groups, having a net charge of zero. This pH is the isoelectric point, which

varies according to the R group involved. An amino acid at its isoelectric point will not migrate under the influence of an applied electric field; it also exhibits a minimum in solubility. Knowledge of the isoelectric point can be used in developing processes for protein purification (Bailey and Ollis, 2nd edition, 1986; Kargi, 1992).

2.1.2 Production of Amino acids

In 1950, all L-Amino acids were used to be manufactured by isolation from natural products and protein hydrolysates or by separation of L-amino acids from the chemically synthesised racemic mixtures (Kirk and Othmer, 1994) . Favorite sources of amino acids have been wheat gluten, blood, casein, and hair. A certain amount of amino acids are still derived from natural sources today (McKetta, 1977). Since 1950, methods of production of L-amino acids have changed a lot, due to the invention of a new fermentation process using *Corynebacterium glutamicum* and *Brevibacterium flavum* for L-glutamic acid production. With the development of fermentation processes, most amino acids are now economically produced via fermentation. Subsequent to the developments of fermentative manufacture of amino acids, enzymatic processes were developed to produce L-aspartic acid, L-cysteine, L-serin, L-phenylalanine, L-tyrosine, L-tryptophan, L-lysine and some D-amino acids from chemically synthesised substrates.

Chemical production is still used on large scale synthesis of glycine, alanine and methionine. However, chemical synthesis of the amino acids has the disadvantage of being expensive and particularly inefficient because it makes a mixture of both L- and D-isomers. Microbial production yields only biologically active L-isomer and therefore seems to be a more promising way of amino acid production (Torres et al., 2000).

The most common industrial examples of the fermentative amino acid productions are the following:

1.Wild/mutant type strain for glutamic acid, glutamine, alanine production
2.Auxotrophic mutants for lysine, ornithine, threonine, tryptophan production
3.Regulatory mutants for lysine, isoleucine, phenylalanine production

4.Precursor addition for threonine, isoleucine and tryptophan production (McKetta, 1977).

The pathway for amino acid biosynthesis is regulated at the key enzyme mainly by two regulation mechanisms-feedback inhibition (inhibition of enzyme activity usually by the end product of the pathway) and repression (repression of enzyme formation usually by the end product). These prevent the overproduction of amino acids in the wild-type microbial cells. Amino acid production is attained by selecting favorable culture conditions, and /or changing the bacterial trait by mutational or genetic treatment to overcome the feedback regulations and induce the overproduction and excretion of amino acids outside the cells. Many kinds of amino acids, e.g. L-phenylalanine and L-tyrosine, are accumulated by auxotrophic mutant strains (which are altered to require some growth factors such as vitamins and amino acids). In these mutants, the formation of regulatory effectors on the amino acid biosynthesis is genetically blocked and the concentration of the effectors is kept low enough to release the regulation and induce the overproduction of the corresponding amino acid and its accumulation outside the cells. Apart from auxotrophic mutants, L-tryptophan is also overproduced by the regulatory mutants in which the feedback regulations are genetically released (Kirk and Othmer, 1994)

2.2 Aromatic amino acids

Phenylalanine, tyrosine and tryptophan are the aromatic amino acids. The phenylalanine R group contains a benzene ring, tyrosine contains a phenol group and the tryptophan R group contains the heterocyclic structure, indole. In each case the aromatic moiety is attached to the α-carbon through a methylene (-CH_2-) carbon (T. Devlin, 1997,4th edition).

Generally aromatic ring systems are planar and electrons are shared over the whole ring structure. Thus aromatic amino acids are relatively nonpolar. Tyrosine is the only the aromatic amino acids with an ionizable side chain, due to containing a hydroxyl group. Phenylalanine is the most hydrophobic aromatic amino acid. The hydrophobic character of tyrosine and phenylalanine is hampered by the existence of polar groups at the side chain. The

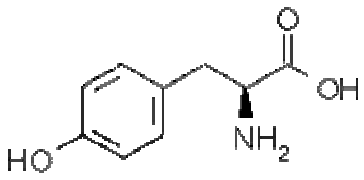
production, usage area, prices and methods of production of the aromatic amino acids are given in Table 2.1.

Table 2.1 Production and Price of Aromatic Amino Acids (Kirk and Othmer, 1994)

no acid	Method of synthesis	Uses	Price, $/kg	Production, ton/year
L-Trp	Chemical, enzymatic synthesis, fermentation	In medicine , especially in the design of antidepressants and sleep-inducers.	74-147	250
L-Phe	Chemical, enzymatic synthesis, fermentation	In food supplements	59-88	3000
L-Tyr	Extraction, fermentation	In food supplements and medicine	74-110	60

2.2.1 Tyrosine

L-Tyrosine (Tyr) is a polar, hydrophilic, weakly acidic amino acid. Its isoelectric point is pI=5.66. The structure of tyrosine is given in Figure 2.3. Tyrosine is abbreviated as Tyr and y.



tyr y Tyrosin

Figure 2.3 Tyrosine

Tyr is an nonessential amino acid as it is synthesized in the body by the hydroxylation of phenylalanine. It is the first product in phenylalanine degradation. Tyr is the precursor of some important neurotransmitters like

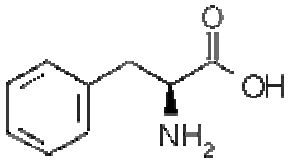
epinephrine, norepineprine and dopamine. It is also the precursor of dihydroxyphenylalanine (L-DOPA); which is later converted to melanin pigments (Bohinski, 5th edition,1987). Tyr is therapeutic in some cases of stress and depression. In addition there has been research in evaluating whether various psycopathologies (such as schizophrenia) are associated with abnormally high levels of tyrosine or its catabolism products (Bohinski, 5th edition,1987). Tyr also participates in the synthesis of thyroid hormone, melanin and quinoproteins that are used in oxidation-reduction reactions (Devlin, 1997).

As Tyr binds unstable molecules (called free radicals), it is considered to be a mild antioxidant. Thus it may be useful for people who have been exposed to harmful chemicals and radiation (http:// www. healthandage. com/ html/ res/ com/ ConsSupplrments / Tyrosinecs.html).

Tyr is produced from *Corynebacterium glutamicum* and *B. lactofermentum*. It has been reported that L-tyrosine with a 9 kg m^{-3} yield is produced by mutant *Corynebacterium glutamicum*, whose gene donor is *E.coli* (Moses and Cape, 1994; Kirk and Othmer, 1994). Mutants resistance to m-fluorophenylalanine have been used in fermentation to give 2.2 kg m^{-3} Tyr in 70 h. Phenylalanine-requiring organisms with n-paraffins (C_{12} –C_{14}) as substrate gave 3.1 kg m^{-3} tyrosine in 4 days. Hagino and Nakayama (1973) were able to select organisms that could accumulate 17.6 kg m^{-3} tyrosine in 96 h on a cane molasses medium; by combining auxotrophy and analog resistance. Other polyauxotrophic mutants were selected by the same group resulting a Tyr accumulation of 15 kg m^{-3} in 72 h using 20% sucrose medium (McKetta,1977).

2.2.2 Phenylalanine

L-Phenylalanine (Phe) is a neutral, nonpolar, amino acid. Its isoelectric point is pI=5.48. The structure of phenylalanine is given in Figure 2.4. It can also be named as Phe and f. Phe is an essential amino acid for the development and maintenance of humans as well as the agriculturally important animals.



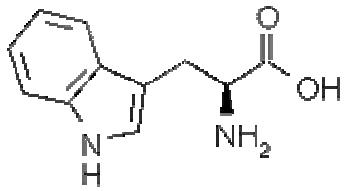
phe f Phenylalanin

Figure 2.4 Phenylalanine

In molasses media, Hagino and Nakayama (1974) achieved 9.5 kg m^{-3} Phe in 96 h with mutants of *C. Glutamicum*. A Tyr auxotroph of hydrocarbon-utilizing bacterium accumulated 10 kg m^{-3} phenylalanine in 68 h from n-alkanes. Peak yields were strongly related to pH (6 being the optimum) and to the addition of 0.7 % corn-steep liquor (10 kg m^{-3} phenylalanine attained in 44 h with Tyr and corn-steep additions). Tryptophan (Trp) also enhanced Phe production. An Ajinomoto patent reports on use of acetic acid or methanol as substrate; reaching 16 kg m^{-3} Phe in 48 h (Kirk and Othmer, 1994; McKetta,1977). Phe is also produced from *Brevibacterium lactofermentum* (Moses and Cape, 1994).

2.2.3 Tryptophan

L-Tryptophan (Trp) is a neutral, nonpolar, amino acid. Its isoelectric point is pI=5.89. The structure of tryptophan and different nomenclatures given to it can be seen in Figure 2.5. Trp, an essential amino acid, is the precursor to serotonin, which is a necessary neurotransmitter responsible for transmitting nerve impulses in the brain and inducing sleep. Serotonin deficiencies, which are related to Trp deficiencies, are well documented in the case of depression and insomia. Trp deficiency also causes pellagra.



trp w Tryptophan

Figure 2.5 Tryptophan

L-tryptophan is generally chemically synthesised, which has the disadvantage of being expensive and particularly inefficient because it makes a mixture of D- and L-isomers. In addition to chemical synthesis, it has been produced by transformation from L-serine and indole catalyzed by *Escherchia coli*, immobilized in K-carrageenan gel beads in the liquid-impelled loop reactor, using organic solvent (Mateus et al., 1996). Related with the production by fermentation, Trp is generally produced by genetically modified strains of *E.coli* and *Corynebacterium glutamicum*, *Brevibacterium flavum* (Devlin, 4th edition, 1997 ; Moses and Cape, 2nd edition, 1994). Table 2.2 gives most common Trp producers.

Trp has been produced via fermentation when anthranilic acid or indole or indole derivatives were added as precursors. With glucose only (no precursor), Trp levels of 6 kg m^{-3} were noted, but with continuous feed of indole, anthranilic acid and nutrients 14 kg m^{-3} Trp production were reported in a 10-day run using *Hansenula anomala*. Hydrocarbon substrates are suggested for Trp production. (McKetta,1977). Azuma et al., (1993) isolated L-tryptophan producing microorganism from *Escherchia coli* W3110 trpAE1 trpR tnaA, which carried pSC101-trpI15.14. They reported that after certain L-tryptophan concentration, indole began to appear in the broth, and the emergence of indole was caused by inhibition of tryptophan synthase due to the accumulated L-tryptophan.

Table 2.2 L-Tryptophan production in fermentation by various microorganisms (Atkinson & Mavituna, 1991)

Microorganism	Titre ($kg\ m^{-3}$)	Productivity ($kg\ m^{-3}\ h^{-1}$)
Claviceps purpurea	1.5	0.010
Escherchia coli	0.7	0.015
Hansenula anomala	14	0.054
Bacillus subtilis	6.2	0.128
Escherchia coli	1.3	0.080
Escherchia coli	6.2	0.229
Brevibacterium flavum	11.4	-

An addition of non-ionic detergents into the supernatant was effective for decreasing the solubility of L-tryptophan, hence causing crystallization of L-tryptophan consequently increase in its production. Kawasaki et al., (1996) reported L-tryptophan production by a pyruvic acid-producing *E. coli* carrying the tryptophanase structural gene from *Enterobactera aerogenes* downstream of the tac promoter. By addition of ammonia and indole to the culture medium containing pyruvic acid, L-tryptophan had been produced. Marin-Sanguino and Torres (2000) have applied the indirect optimization method to the maximization of tryptophan production. Dodge and Gernstner (2002) , by using an engineered strained of E.coli, developed a fed-batch fermentation process and increased the yield on glucose to 0.176 producing 42.3 $kg\ m^{-3}$ Trp.

2.2.4 Aromatic Amino Acid Synthesis Pathway

Aromatic amino acid synthesis pathway has been engineered successfully for the synthesis of natural and unnatural chiral amino acids that are important drug intermediates; as well as other industrially important aromatics such as indigo. Production of aromatics via engineered microorganisms provides environmental and economical advantages like exclusive use of aqueous solvent, non-toxic intermediates and lower material cost. Thus intense interest has developed in the enzymes of this metabolic pathway

(Monbouquette and Liao, http://www.wtec.org/me/me2001/Monbouquette.pdf).

The first step of aromatic amino acid biosynthesis pathway begins with condensation between PEP (phosphoenolpyruvate) and E4P (erythrose-4-phosphate) which are products of pentose phosphate pathway and glycolysis as it is seen in Figure 2.6.

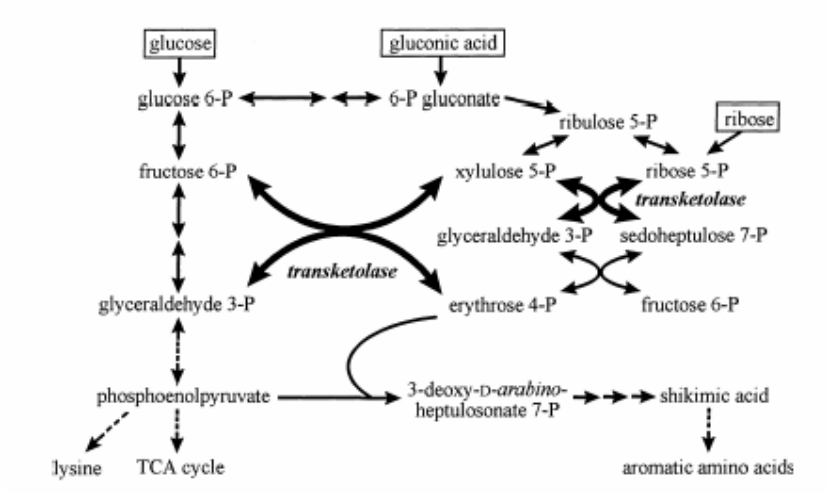


Figure 2.6 The pentose phosphate pathway, glycolysis and aromatic biosynthesis pathway in bacteria. Dashed lines multiple enzymatic steps (M.Ikeda, K. Okamoto, R. Katsumata, 1999).

An important intermediate, at the branch point between the specific routes of the three amino acid biosynthesis is chorismate (Figure 2.7). Both tyrosine and phenylalanine are derived from chorismate, the carbons of which can be traced back to E4P and PEP.

2.3 Bioprocess Parameters in Amino Acid Production

Bioprocess engineering is the application of engineering principles to design, develop and analyze processes using biocatalyst for the production of a desirable product or for the destruction of unwanted or hazardous substances.

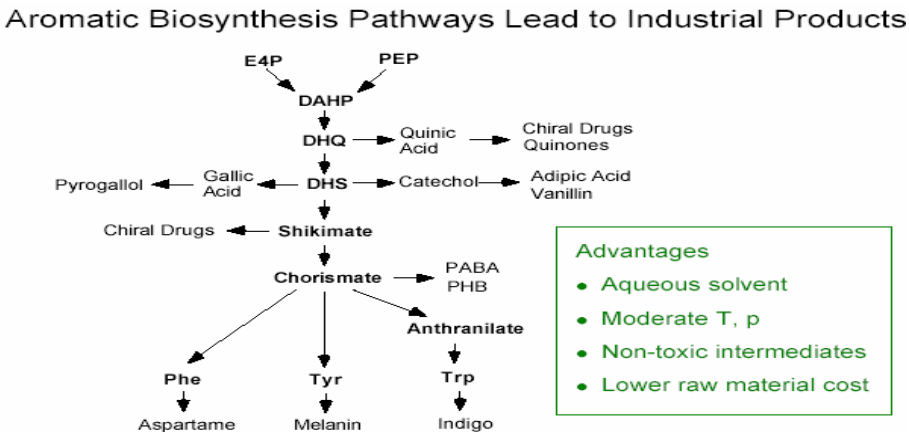


Figure 2.7 Aromatic amino acid pathway

In this context microorganism, the medium, agitation and air inlet rate of the bioreactor, pH and finally the temperature are very important parameters in the design of a bioprocess. The investigation of these bioprocess operation parameters and their simultaneously optimization for the maximum yield of the product are very important.

2.3.1 Microorganism

In bioprocesses the first important step is to select the most proper microorganism for the production of the product, screening a wide range of

candidate microorganisms. The microorganism should be able to secrete large amount of the desired product using cheaper substrates, without producing toxins or any other harmful products (Kirk and Othmer, 1994).

In this study, *Bacillus acidocaldarius* NRRC-207 F was used for the investigation of aromatic amino acid production.

2.3.1.1. *Bacillus acidocaldarius*

Bacillus acidocaldarius is an aerobic , thermo-acidophilic and chemoheterotroph bacterium (Darland and Brock, 1971). Being a chemoheterotroph bacterium, it can obtain energy from the oxidation of inorganic compounds. It is a rod-shaped, gram positive microorganism that possess unique fatty acids (-cyclohexane or –cycloheptane fatty acids) as the major components of the cellular membrane (Goto et al., 2002). It is also called as *Alicyclobacillus acidocaldarius.* The organism survives under extreme environmental conditions at temperatures between 45-90°C and extremely acid pH values (pH 2-5). Their optimum growth temperature is about 60°C and optimum pH is about 3 to 4 (Darland and Brock, 1971). DNA base composition of this microorganism is 62 to 64 moles % guanine+cytosine. This number is generally much higher than those of the other microorganisms. It is also characterized by endospore-formation and the possession of ω-alicyclic fatty acid as a major cellular fatty acid-probably causing the maintenance of organizational integrity of the cell contents in these extreme conditions (Hauser et al., 2000). *Alicyclobacillus acidocaldarius* is generally considered to inhabit soil-both geothermal and nongeothermal soils- and to contaminate and grow occasionally in fruit juices (Matsubara et al., 2002). *Alicyclobacillus acidocaldarius* maintains its cytoplasmic pH at a value close to neutrality irrespective of the external pH (Langworthy 1979). Thus the structural properties of its membrane-containing fatty acids (-cyclohexane or –cycloheptane fatty acids) - are such that the microorganism can tolerate large trans-membrane pH gradient (Farrand et al., 1983) - ΔpH values upto 5 units across their cell membrane. The maintenance of ΔpH is an

active process, driven by outwardly pumping of protons across the cell membrane, and the subsequent exchange against inwardly moving K^+ ions. Hence, the large proton-motive force of acidophiles consists of a large ΔpH component and a low membrane potential. Below pH 3, membrane potential reverses its sign from internally negative to internally positive. In this respect this micrroorganism is unique in that it can maintain a positive membrane potential .The result of this strategy is that only those cell macromolecules that face the medium have to adapt to acidic conditions (Bakker et al., 1997)

2.3.1.1 Growth Kinetics, Product Formation and Yield Coefficients

Microbial growth involves many complex chemical reactions occurring inside the cells. These reactions are transport of substrates into the cell, conversion of the intracellular substrates into biomass and metabolic products and then the excretion of metabolic products into the extracellular medium.

Rate of microbial growth is directly proportional to cell concentration. The specific growth rate, μ, is defined as the following:

$$\mu = \frac{1}{C_x}\frac{dC_x}{dt} \tag{2.1}$$

where C_X is the cell mass concentration (kg m^{-3}), t is time (h), and μ is the specific growth rate (h^{-1}) (Shuler and Kargi, 1992).

Several phases of cell growth are observed in batch culture. Table 2.3 provides a summary of batch cell growth and metabolic activity during the phases of batch culture (Doran, 1995).

During the exponential growth period- including lag, acceleration and growth phases-in batch growth, rate of cell growth, r_X, is described by the following equation (Shuler and Kargi, 1992) :

$$r_X = \frac{dC_X}{dt} = \mu C_X \tag{2.2}$$

Table 2.3 Summary of batch cell growth

Phase	Description	Specific growth rate
Lag	Cells adapt to the new environment	$\mu \approx 0$
Acceleration	Growth starts	$\mu < \mu max$
Growth	Growth achieves its maximum rate	$\mu \approx \mu max$
Decline	Growth slows due to nutrient exhaustion or build-up of inhibitory products	$\mu < \mu max$
Stationary	Growth ceases	$\mu = 0$
Death	Cells lose viability and lyse	$\mu < 0$

Similarly, substrate consumption rate, r_S and product formation rate, r_P, are described by the following equations respectively where C_S is the substrate concentration and C_P is the product concentration:

$$r_S = -\frac{dC_S}{dt} \tag{2.3}$$

$$r_P = \frac{dC_P}{dt} \tag{2.4}$$

Similarly, specific product formation rate, q_P, and specific substrate consumption rate, q_S, are described by the following equations respectively:

$$q_p = \frac{1}{C_X}\frac{dC_P}{dt} \tag{2.5}$$

$$q_S = -\frac{1}{C_X}\frac{dC_S}{dt} \tag{2.6}$$

To better describe growth kinetics, some stoichiometrically related parameters, namely yield coefficients, are defined. As a general definition,

$$Y'_{P/S} = \frac{-\Delta P}{\Delta S} \tag{2.7}$$

where, $Y'_{P/S}$ is the observed overall yield coefficient, P and S are product and substrate, respectively, involved in metabolism. ΔP is the mass or moles of P produced, and ΔS is the mass or moles of S consumed. This definition gives an overall yield representing some sort of average value for the entire culture period. However, in batch processes, the yield coefficients may show variations throughout the process for a given microorganism in a given medium, due to the growth rate and metabolic functions of the microorganism. Therefore, it is sometimes necessary to evaluate the instantaneous yield at a particular point in time. Instantaneous yield can be calculated as follows:

$$Y_{P/S} = \frac{-dP}{dS} = \frac{-dP/dt}{dS/dt} = \frac{r_P}{r_S} \tag{2.8}$$

When yields for fermentation are reported, the time or time period to which they refer should be stated (Doran, 1995). A list of frequently used yield coefficients is given in Table 2.4.

To understand the above mentioned variations in yield coefficients, it is useful to breakdown substrate consumption into three parts: provision of energy for cell synthesis, provision of energy for product synthesis and provision of energy for maintenance. Maintenance here refers to the collection of cell energetic requirements for survival or for preservation of a certain cell state, which are not directly related to or coupled with the synthesis of more cells. Such activities include active transport of ions and other species across cell membranes and replacement synthesis of decayed cell constituents, regulation of internal pH, DNA replication etc…(Bailey and Ollis, 1986).

Table 2.4 Definition of yield coefficients.

Symbol	Definition	Unit
$Y'_{X/S}$	Mass of cells produced per unit mass of substrate consumed	g cell g^{-1} substrate
$Y'_{X/O}$	Mass of cells produced per unit mass of oxygen consumed	g cell g^{-1} oxygen
$Y'_{S/O}$	Mass of substrate produced per unit mass of oxygen consumed	g substrate g^{-1} oxygen
$Y'_{P/X}$	Mass of product formed per unit mass of cell produced	g product g^{-1} cell
$Y'_{P/S}$	Mass of product formed per unit mass of substrate consumed	g product g^{-1} substrate
$Y'_{P/O}$	Mass of product formed per unit mass of oxygen consumed	g product g^{-1} oxygen

The maintenance coefficients for oxygen denoted by m_0, is oxygen consumption for maintenance. Oxygen is consumed for roughly three purposes: product formation, cell growth and for maintenance. In this context, $-r_{01}$, $-r_{02}$, $-r_{03}$ are defined as the following where $\overline{Y}$ is the true overall yield coefficient-differentiating energy consumption for maintenance from energy consumption from product or cell formation :

$$-r_{01} = \frac{dC_X / dt}{\bar{Y}_{X/0}} \quad , \tag{2.9}$$

oxygen consumption rate for cell growth.

$$-r_{02} = \frac{dC_P / dt}{\bar{Y}_{P/0}} \quad , \tag{2.10}$$

oxygen consumption rate for product formation.

$$-r_{03} = m_0 C_X \quad , \tag{2.11}$$

oxygen consumption rate for cell maintenance.

Thus, total oxygen consumption rate, $-r_0$, is the following:

$$-r_0 = (-r_{01}) + (-r_{02}) + (-r_{03}) \tag{2.12}$$

Equations (2.9), (2.10) and (2.11) are substituted into (2.12) to obtain,

$$-\frac{dC_O}{dt} = -r_0 = (1/\bar{Y}_{X/0})\frac{dC_X}{dt} + (1/\bar{Y}_{P/0})\frac{dC_P}{dt} + m_0 C_X \tag{2.13}$$

Putting (2.2) and (2.5) into (2.13),

$$-r_0 = (1/\bar{Y}_{X/0})\mu\, C_X + (1/\bar{Y}_{P/0})\, q_P\, C_X + m_0\, C_X \tag{2.14}$$

Dividing equation (2.14) by C_X

$$\frac{-r_0}{C_X} = \frac{\mu}{\bar{Y}_{X/0}} + \frac{q_P}{\bar{Y}_{P/0}} + m_0 \tag{2.15}$$

In order to find maintenance coefficient, specific product formation rate, q_P, should be related with specific growth rate, μ. For this reason, in this study, the model presented by Leudeking -Piret was used in the rough-estimation of maintenance coefficients. The model is the following:

$\frac{dC_P}{dt} = \alpha \frac{dC_X}{dt} + \beta C_X$, where α and β are Leudeking-Piret constants of a bioprocess (Bailey and Ollis, 1986). (2.16)

Dividing Eqn. (2.16) to C_X,

$$q_P = \frac{1}{C_X}\frac{dC_P}{dt} = \alpha\mu + \beta \quad (2.17)$$

In this equation α is the term for growth associated product formation and β is the term for non-growth associated product formation. Adding equation (2.17) into (2.15),

$$\frac{-r_0}{C_X} = \frac{\mu}{\bar{Y}_{X/O}} + \frac{\alpha\mu + \beta}{\bar{Y}_{P/O}} + mo \quad (2.18)$$

Dividing (2.18) to μ,

$$\frac{-r_0}{\mu C_X} = \frac{1}{\bar{Y}_{X/0}} + \frac{\alpha + (\beta\mu^{-1})}{\bar{Y}_{P/O}} + \frac{mo}{\mu} \quad (2.19)$$

Rearranging (2.19),

$$\frac{-r_O}{\mu C_X} = \frac{-r_0}{dC_X / dt} = \frac{\frac{-dC_O}{dt}}{\frac{dC_X}{dt}} = \frac{1}{Y_{X/0}} = (\frac{\alpha}{\bar{Y}_{P/O}} + \frac{1}{\bar{Y}_{X/O}}) + (\frac{1}{\mu})(mo + \frac{\beta}{\bar{Y}_{P/O}}) \quad , \qquad (2.20)$$

From the slope of the plot of ($1/Y_{X/0}$)versus (1/μ) , ($mo + \frac{\beta}{\bar{Y}_{P/O}}$) (g oxygen g^{-1} dry cell weight h^{-1}) and from the intercept, $(\frac{\alpha}{\bar{Y}_{P/O}} + \frac{1}{\bar{Y}_{X/O}})$ could be determined; where Y presents the instantaneous yield and $\overline{Y}$ presents the true overall yield and Y' presents the observed overall yield.

With the assumption of $(\frac{Y'_{P/O}}{Y'_{X/O}} \approx \frac{\bar{Y}_{P/O}}{\bar{Y}_{X/O}})$ for the period of investigation, $(\frac{\bar{Y}_{P/O}}{\bar{Y}_{X/O}})$ can be determined. Thus mo can be calculated.

Similarly, the maintenance coefficients for substrate denoted by m_S could be determined. m_0 and m_S may differ with the change in bioprocess parameters such as, type of microorganism, type of substrate, pH and temperature.

2.3.2 Medium Design

Many factors must be considered when designing a fermentation medium. Nutrients (reactants) must be provided in sufficient quantities and proper proportions for a specified amount of biomass and product(s) to be synthesized. Computation of the necessary amounts of various substrates clearly requires knowledge of the product and/or biomass composition. Also important in medium formulation is provision of necessary minerals like phosphorus, sulphur, potassium, magnesium, sodium, calcium, iron, copper, manganese and molybdenum. Many commercially important microorganisms need simple sugars. Instead of purified, simple sugars, crude sources such as beet, cane or corn molasses are frequently used as carbon and energy

sources in industrial fermentation media. A variety of possible nitrogen sources are available including ammonia, urea and nitrate. If the microorganism produces proteolytic enzymes, however, it can obtain necessary nitrogen from a variety of relatively crude proteinaceous sources. Among the possibilities for such crude sources are cereal grains, peptones, meat scraps, soybean meal, casein, yeast extracts, corn-steep liquor, peanut-oil meal. Also some microorganisms might need external source of some amino acids and growth factors. In industrial practice growth factors are typically provided by some of the crude medium components already mentioned, e.g., corn-steep liquor of yeast autolysate. Similarly, these crude preparations often supply many of the minerals necessary for cell function. pH of the medium is also a very important parameter of medium design.

When product formation is the major target, precursors may be added to the medium to improve the yield or quality. Generally the precursor molecule or a closely related derivative is incorporated into the fermentation product molecule. Specific examples of precursor applications include benzoic acids for production of novobiocins, phenylacetic acid for the manufacture of penicillin G and 5,6-dimethylbenzimidazole for vitamin B_{12} fermentation (Bailey and Ollis, 1986).

In the literature there is no work on systematic investigation of the effects of medium components for aromatic amino acid production by *Bacillus acidocaldarius.* But it has been stated that alicyclobacillus species, including alicyclobacillus acidocaldarius, grows well in apple juice, citrus juice, white grape juice and various blends of juices (Splittstoesser et al., 1994; Splittstoesser et al., 1998). The bacteria found from citrus processing lines are potential spoilage agent for beverages containing citrus juices (Wisse and Parish et al., 1998).

For optimal growth of *Bacillus acidocaldarius*, Farrand et al. (1983) optimised a medium containing (kg m^{-3}): $(NH_4)_2SO_4$ 1.5; KH_2PO_4 1.0; $MgSO_4.7H_2O$ 0.2; $CaCl_2.2H_2O$ 0.1; nitriloacetic acid (NTA) 0.06; $FeSO_4.7H_2O$ 0.01; glucose up to 8.4; and (ml L^{-1}): trace element solution 1.0; polypropylene glycol (Shell, MW 2000) 0.1. This trace element solution contained (kg m^{-3}): $CaCl_2.2H_2O$ 0.66; $ZnSO_4.7H_2O$ 0.18; $CuSO_4.5H_2O$ 0.16; $MnSO_4.4H_2O$ 0.15; $CoCl_2.6H_2O$ 0.18;

H_3BO_3 0.10; $Na_2MoO_4.2H_2O$ 0.3. For Trp production with *E.coli* , the following medium was used (kg m^{-3}): 50 glucose, 10 casamino acid, 0.5 anthranilic acid, 0.01 tetracycline, 3 K_2HPO_4 ,7 K_2HPO_4 , 3 NH_4Cl, 0.2 $MgSO_4.7H_2O$, with pH_O=7 with 2N NaOH. Also a molasses medium was used for Trp production for *Corynebacterium glutamicum*. This medium contained the following :10 % reducing sugars as invert (cane molasses), 0.05 % KH_2PO_4, 0.05 % K_2HPO_4 , 0.025 % $MgSO_4.7H_2O$, 2 % $(NH_4)_2SO_4$, 1% corn steep liquor and 2% $CaCO_3$, with pHo=7.2 (Nakayama,1985; Atkinson and Mavituna, 1991).

2.3 Bioreactor Operation Parameters

Oxygen transfer, pH and temperature are the major bioreactor operation parameters, showing diverse effects on product formation in aerobicfermentation processes by influencing the metabolic pathways and changing metabolic fluxes (Çalık et al., 1999).

2.4.1 Temperature

Temperature is a very important parameter affecting the growth rate of the microorganism, thus affecting the substrate utilization rates and product formation rates; changing the product quality and yield. For every process there is an optimum temperature for maximum product formation. For *Bacillus acidocaldarius,* optimum growth temperature range was found to be 60-65° C (Darland et al., 1971; Nicolaus et al., 1998). In a continuous culture, highest growth yields of *Bacillus acidocaldarius* were obtained at 51°C and pH>4.3 at a dilution rate of 0.1 h^{-1} ($Y_{glucose}$ = 23.4 gcells.mol glucose^{-1} , Y_{O2} =5.9 gcells.mol O_2^{-1}) (Farrand et al., 1983).

2.4.2 pH

Although hydrogen ion concentration of the extracellular broth (fermentation medium) changes according to the metabolic activities of the cell, the intracellular pH of the cell remains constant. The proton gradient between extracellular and intracellular medium is necessary for the energy production

through the transport of the materials and also for the intracellular reaction network (Nielsen and Villadsen, 1994). Extracellular pH changes the metabolic activities of the cell, changing the product yield and quality. And sometimes it might be necessary to control the pH of the medium throughout the fermentation. Thus finding the optimum medium pH and deciding on whether to control pH of the medium or not are very important.

Optimum growth pH of *Bacillus acidocaldarius* was found in the pH range of 3-4 without control (Darland et al., 1971; Nicolaus et al, 1998). In the literature, it was stated that the cytoplasmic pH of this bacteria is kept at a value close to neutrality although it can tolerate low extracelular medium pHs by the help of the special proton motive mechanisms of its membrane (Bakker et al., 1996).

2.4.3 Oxygen Transfer

As microorganisms require oxygen for their metabolic activities like respiration, growth and product formation in aerobic fermentations, the concentration of the dissolved oxygen in the medium and oxygen uptake rate are very important parameters (Çalık et al., 1998). In aerobic organisms, there exists a respiratory chain which involves an oxidative process involving molecular oxygen. This respiration reaction sequence is also known as electron transport chain. In the final phase oxygen is the final electron acceptor. Namely, electrons (or the H atoms) carried by NADH are transferred to oxygen via a series of intermediate compounds (respiratory chain). The energy released from this electron transport results in the formation of up to three ATP molecules. Thus, the major role of electron transport chain is to regenerate NAD(s) for glycolysis and ATP(s) biosynthesis (Kargi, 1992). The deficiency or surplus of the oxygen in the medium affects the metabolism and thus the yield of product. Oxygen necessity of a bioprocess is related with the microorganism, physical properties of production medium, bioreactor and its stirrer style. Agitation and air flow rate strongly affect transfer of the oxygen to the microorganism. Agitation not only changes oxygen transfer rate, but also affects assurance of an adequate travel of nutrients to the cells, efficient heat transfer, accurate measurement of specific metabolites in the cell culture fluid and efficient dispersion of added

solutions such as antifoam agents (Glick and Pasternak, 1998). Too much agitation must be avoided not to damage the cells with shear stress. In the literature, for optimum growth of *Bacillus acidocaldarius,* the stirrer was agitated at a rate of 600 rpm with a working volume of 2L Biotech bioreactor and the aeration was carried out by passing sterile air through it at 0.122 L/min (Farrand et al., 1983).

As the solubility of the oxygen in water is very low, the controlling mechanism of the diffusion of the oxygen in the water is liquid film resistance. Thus the overall mass transfer coefficient, K_La, is approximately equal to the liquid phase mass transfer coefficient, k_La. Thus oxygen transfer rate (OTR) from gas to liquid is given by the following equation:

$$OTR = k_L a(C^*_{o2} - C_{o2}) \tag{2.21}$$

Oxygen uptake rate (OUR) ,r_O, is defined as the rate of oxygen consumption of the microorganism per unit volume of the broth. Specific oxygen uptake rate, q_O , is defined as the rate of oxygen consumption of the microorganism per unit cell concentration.

$$q_O = \frac{OUR}{C_X} \equiv \frac{r_O}{C_X} \tag{2.22}$$

The rate of oxygen transfer in fermentation broths is influenced by temperature, physical, chemical and rheological properties of the media, agitation rate, antifoam agents, the solutes and cells.

2.4.3.1 Measurement of K_La: Dynamic Method

This widely used, simple method can be applied during the fermentation process to determine the value of K_La experimentally. The method is based on an unsteady state mass balance for oxygen given by the following equation:

$$\frac{dC_O}{dt} = K_L a(C_O^* - C_O) - q_0 C_X \tag{2.23}$$

As shown in Figure 2.8 , at some time t_0, the broth is de-oxygenated by stopping the air flow. During this period, dissolved oxygen concentration, Co_0, drops and since there is no oxygen transfer, equation (2.23) becomes:

$$\frac{dC_O}{dt} = r_0 \tag{2.24}$$

Using equation (2.24) in region-II of Figure 2.8, oxygen uptake rate, $-r_0$, can be determined.

Air inlet is then turned back on and the increase in C_O is monitored as a function of time. In this period, region-III, equation (2.23) is valid. Combining equations (2.23) and (2.24) and rearranging,

$$C_O = -\frac{1}{K_L a}\left(\frac{dC_O}{dt} - r_0\right) + C_O^* \tag{2.25}$$

From the slope of a plot of C_O versus ($dC_O/dt - r_0$), K_La can be determined (Figure 2.9).

The dynamic method can also be applied to conditions under which there is no reaction, i.e., $r_0=0$ (Nielsen and Villadsen, 1994). In this case, the broth is de-oxygenated by sparging nitrogen into the vessel. Air inlet is turned back on and again the increase in Co is monitored as a function of time. Modifying equation (2.25)

$$C_O = -\frac{1}{K_L a}\frac{dC_O}{dt} + C_O^* \tag{2.26}$$

From the slope of a plot of C_O versus dC_O/dt, the physical mass transfer coefficient, K_La_0, can be determined.

In the literature, there is no work reporting the oxygen transfer characteristics ($-r_0$, K_La) of the process of aromatic amino acid production by *Bacillus acidocaldarius* species.

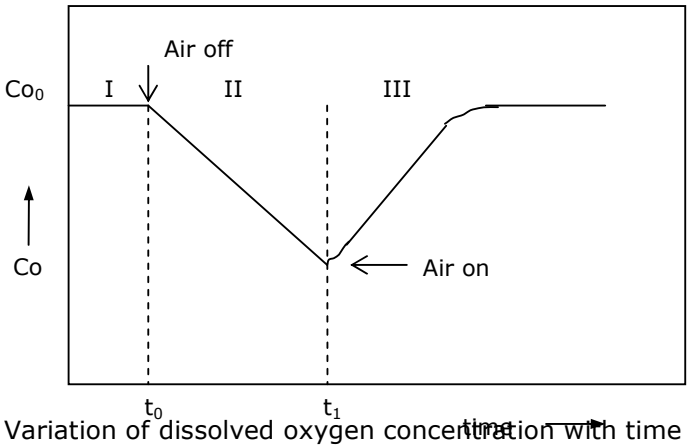


Figure 2.8 Variation of dissolved oxygen concentration with time in dynamic measurement of K_La

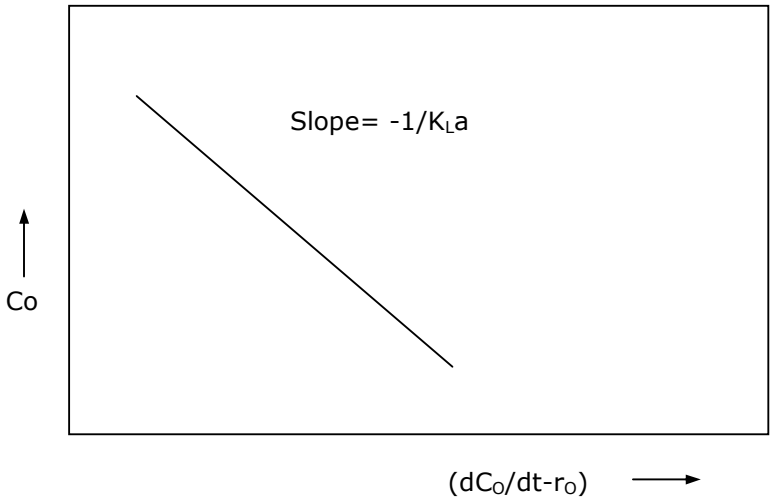


Figure 2.9 Determining K_La using the dynamic method.

CHAPTER 3

MATERIALS AND METHODS

3.1 The Microorganism

Bacillus acidocaldarius NRRC-207 F was used as the potential producer of aromatic amino acids. The microorganisms, which were freeze dried when received, were kept at -20ºC and brought to an active state by incubating for 30 min, at 30ºC, in a liquid medium, V=0.3ml, that contained (kg m^{-3}): 1, Yeast extract; 0.2, $(NH_4)_2SO_4$; 0.5, $MgSO_4$ x 7 H_2O; 0.186 ,$CaCl_2$;0.6, KH_2PO_4 ; 1, glucose; 25, agar . Afterwards, the microorganisms were inoculated onto a solid medium and stored at 4ºC.

3.2 The Solid Medium

The microorganisms, stored on agar slants at 4ºC, were inoculated onto the freshly prepared agar slants under sterile conditions and were incubated at 55ºC for 12h; thereafter inoculated into the pre-cultivation medium. The composition of the solid medium for growth of the bacteria is given in Table 3.1.

Table 3.1 The composition of the solid medium for *Bacillus acidocaldarius*.

Compound	Concentration, kg m^{-3}
Yeast extract	1.0
$(NH_4)_2SO_4$	0.2
$MgSO_4$ x 7 H_2O	0.5
$CaCl_2$	0.186
KH_2PO_4	0.6
Glucose	1.0
Agar	25.0

3.3 The Precultivation Medium

Microorganisms grown in the solid medium were inoculated into precultivation medium and incubated at 55ºC and N=175 min^{-1} for 8-9 h. Experiments were conducted in agitation and heating rate controlled orbital shakers, using air-filtered Erlenmeyer flasks 150 ml in size that had working volume capacities of 33 ml. The composition of the precultivation medium for cell growth is given in Table 3.2

3.4 The Production Medium

When the microorganism concentration in the precultivation medium reached to 0.43 kg m^{-3}, the microorganisms were inoculated to the production medium, contained either in the laboratory scale bioreactor (V=150 ml) or pilot scale bioreactor (V=3.0 dm^3), with 1/10 inoculation ratio.

Table 3.2 The composition of the precultivation medium for *Bacillus acidocaldarius*

Compound	Concentration, kg m^{-3}
Yeast extract	1.96
$(NH_4)_2SO_4$	0.65
$MgSO_4$ x 7 H_2O	0.48
$CaCl_2$	0.179
KH_2PO_4	2.88
Glucose	9.8

For laboratory scale experiments, air-filtered Erlenmeyer flasks 150 ml in size, that had working volume capacities of 33 ml, were used as bioreactors. Agitation and heating rate controlled orbital shakers, at a cultivation temperature of 55ºC and an agitation rate of 175 min^{-1}, unless otherwise stated, were used as incubators. Details of the pilot scale experiments are given in section 3.5. The parameters investigated in the production medium are given in Table 3.3.

All the medium components except the salt solution were steam sterilized at 121°C for 20 min, glucose and fructose being sterilized separately. The micronutrients all together, referred to as the salt solution, was filter sterilized with a sterile filter of 0.2 µm pore size.

Table 3.3 The investigated parameters

Medium Components	Bioreactor Design Parameters
Glucose	Uncontrolled- and controlled-pH
Fructose	Temperature
Sucrose	Oxygen Transfer Rate
Citric acid	
Maltose	
$(NH_4)_2HPO_4$	
NH_4Cl	
NH_4SO_4	
Acid type H_3PO_4 , HCl, H_2SO_4	

3.5 The Pilot Scale Bioreactor System

The pilot scale 3.0 dm^3 batch bioreactor (Braun CT2-2), having a working volume of 0.5-2.0 dm^3 and consisting of temperature, pH, foam, stirring rate controls, was used. The diameter and height of the bioreactor were 121 mm and 270 mm, respectively. The bioreactor utilized an external cooler, steam generator and a jacket around the bioreactor for sterilization and temperature control. The bioreactor was stirred with two four-blade Rushton turbines and consisted of four baffles, two six-bladed impellers and a sparger. Details of the bioreactor is given in Table 3.4.

Table 3.4 Characteristics of the bioreactor

Diameter of the bioreactor	121 mm
Height of the bioreactor	270 mm
Orifice Diameter	0.5 mm
Number of Orifice	14
Impeller Diameter	53 mm

3.6 Analysis

Throughout the bioprocess, samples were taken at different cultivation times. After determining the cell concentration, the medium was centrifuged at 13000 min^{-1} for 10 min to separate the cells. Supernatant was used for the determination of fructose concentration. In bioreactor system experiment, besides cell and fructose concentrations; amino acid and organic acid concentrations, oxygen uptake rate and liquid phase mass transfer coefficient values were determined.

3.6.1 Cell Concentration

Cell concentrations based on dry weights were measured with a UV-Vis spectrophotometer (Thermo Spectronic, Heλios α) using the calibration curve previously obtained at 600 nm, for *Bacillus licheniformis*, by M. Arifoglu (Appendix A).

3.6.2 Reduced Sugar Concentration

Reduced sugar, glucose, concentration was determined by the DNS (dinitrosalisylic acid) method (Miller, 1959) at 550 nm with a UV spectrophotometer. The calibration curve and the preparation method of the DNS solution are given in Appendix B and C, respectively. The method used in analysis of samples and preparation of the calibration curve is given below:

1. 3 cm^3 of DNS solution was added into test tubes containing 1 cm^3 of sample at different glucose concentrations.

2. The test tubes were put into boiling water for 5 min and then into ice for another 5 min.

3. The sample passing through the same steps but do not contain any reducing sugar is used as blank and the absorbance values of the samples were measured by a UV spectrophotometer at 550 nm.

3.6.3 Amino Acids Concentrations

Amino acid concentrations were measured with an amino acid analysis system (Waters, HPLC), using the Pico Tag method (Cohen, 1983). The method is based on reversed phase HPLC, using a precolumn derivation technique with a gradient program developed for amino acids. The amino acid concentrations were calculated from the chromatogram, based on the chromatogram of the standard amino acids solution. The analysis was performed under the conditions specified in Table 3.5.

3.6.4 Organic Acids Concentrations

Organic acid concentrations were determined with a high performance capillary electrophoresis at 254 nm (Waters HPCE, Quanta 4000E). The samples were analyzed at 20kV and 15°C with a negative power supply by hydrostatic pressure injection, using an electrolyte containing 5mM potassium hydrogen phtalate and 0.5mM OFM Anion Bt (Waters) as the flow modifier at pH=5.6 (for α-ketoglutaric, acetic, malic, fumaric, succinic, lactic, oxalacetate and gluconic acids) and at pH=7.0 (for, pyruvic, citric, lactic, gluconic acids) (Çalık et al., 1998).

Table 3.5 Conditions of the Amino Acid Analysis

Column	:Amino acid analysis column (Nova-Pak C18, Millipore)
Column dimensions	:3.9 mm x 30 cm
System	:Reversed phase chromatography
Mobile phase flow rate	:1 ml/min
Column temperature	:38 °C
Detector and wavelength	:UV/VIS, 254 nm
Injection volume	:4 µl
Analysis period	:20 min

3.6.5 Liquid Phase Mass Transfer Coefficient and Oxygen Uptake Rate

In order to determine the liquid phase mass transfer coefficient and oxygen uptake rate, the Dynamic Method (Rainer 1990), as explained in section 2.4.3, was used.

Prior to inoculation of the microorganism to the production medium in the bioreactor, the physical mass transfer coefficient (K_La_0) was determined. After inoculation of the microorganism to the bioreactor, the dynamic oxygen transfer experiments were carried out at certain cultivation times for a short period of time, so that the biological activities of the microorganisms are unaffected. During this period, while the air inlet was totally ceased, the agitation rate was lowered to N=100 min^{-1} in order to lower the effect of surface aeration.

CHAPTER 4

RESULTS AND DISCUSSIONS

Investigation of aromatic amino acid synthesis performance of *Bacillus acidocaldarius* NRRC-207 F Rev.2/78 was carried out at three steps. Firstly a medium design was carried for finding the optimum medium for maximum cell concentration. Secondly using the optimum medium, the effects of bioprocess operation parameters-pH and temperature- were investigated for best cell growth. Finally the effects of oxygen transfer on aromatic amino acid production capacity and oxygen transfer characteristics were investigated in pilot scale bioreactor.

4.1 Medium Design

In medium design studies, the comparison of the effect of a variable on cell concentration could be made at the time when cells reached their maximum concentrations. As cell concentration mostly reached its maximum concentration at nearly 17 h, cell concentrations were always measured at t=17 h of the bioprocess.

4.1.1 Effect of initial pH

A defined medium was designed which is optimum for cell growth. As the starting point of the defined medium (Ref-1) designed for alkaline protease production (Çalık et al., 2003a) that contained (kg m^{-3}): glucose, 8; $(NH_4)_2HPO_4$, 4.7; $CaCl_2$, 0.2; KH_2PO_4, 2; $NaH_2PO_4.2H_2O$, 7.318; Na_2HPO_4, 0.0438; $Mg(CH_3COO)_2.4H_2O$,87×10^{-3} was used and effects of the pH was

investigated between pH=3-5. The pH of the media were adjusted by sulphuric acid. As it is seen in Figure 4.1, The optimum initial pH for growth was found 5. Cell concentration was measured at t=17 h and C_X = 0.280 kg m^{-3} at pH_0 =5. No cell growth was seen with the medium having an initial pH of 3.

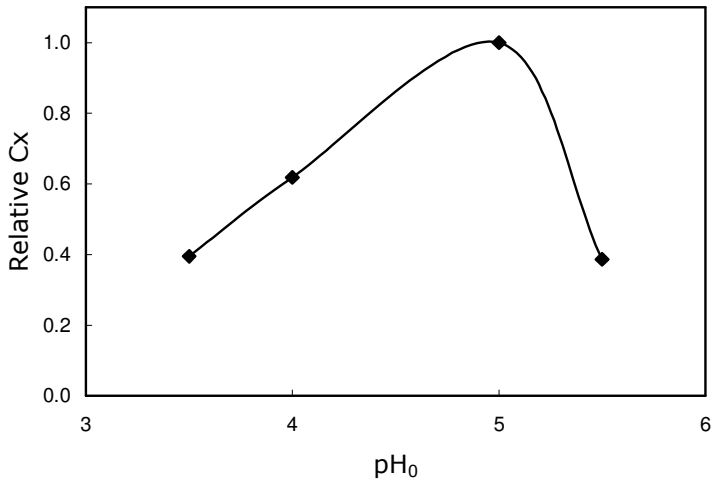


Figure 4.1 The variation in relative cell concentration with the initial pH of the medium, T=55°C and N=175 min^{-1}

4.1.2 The effect of inorganic nitrogen sources on cell growth

To investigate the effect of the inorganic nitrogen sources, Ref-1 medium in which pH of the production medium was adjusted by H_2SO_4 (aq) was used. The effects of $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, NH_4Cl on cell growth were investigated and the initial concentrations were (kg m^{-3}): NH_4Cl, 1.9; $(NH_4)_2SO_4$, 4.707; $(NH_4)_2HPO_4$, 4.71, adjusted such that in each case the amount of nitrogen in the medium was equivalent to the amount of nitrogen in the medium containing 4.71 kg m^{-3} of $(NH_4)_2HPO_4$. It is seen in Table 4.1 that $(NH_4)_2HPO_4$ was found to be the best nitrogen source for the medium in terms of cell growth.

Table 4.1 Effect of Inorganic Nitrogen Sources on Relative Biomass Concentration

Nitrogen Source	Relative Cell Concentration at t=17. h
$(NH_4)_2HPO_4$	1.00
$(NH_4)_2SO_4$	0.71
NH_4Cl	0.82

4.1.3 Effect of $(NH_4)_2HPO_4$ concentration

By using the Ref-1 medium effect of $(NH_4)_2HPO_4$ concentration was investigated at $C_{(NH4)2HPO4}$ = 3, 4, 4.5, 5, 5.5, 6, and 7 kg m^{-3}. As it is seen in Figure 4.2, best cell concentration is at $C_{(NH4)2HPO4}$ = 5 kg m^{-3}.Thus, the medium turned to the following (kg m^{-3}): glucose, 8; $(NH_4)_2HPO_4$, 5; $CaCl_2$, 0.2; KH_2PO_4, 2; $NaH_2PO_4.2H_2O$, 7.318; Na_2HPO_4, 0.0438; $Mg(CH_3COO)_2.4H_2O$, 87×10^{-3} with pH_0 =5, adjusted by H_2SO_4.

4.1.4 Effect of the acid used to adjust initial pH of the medium

In order to adjust the pH of the medium, effects of H_2SO_4, H_3PO_4 and HCl were investigated. The highest cell concentration was obtained with H_3PO_4 (Table 4.2). Therefore, for the pH adjustment H_3PO_4 was selected, for future experiments.

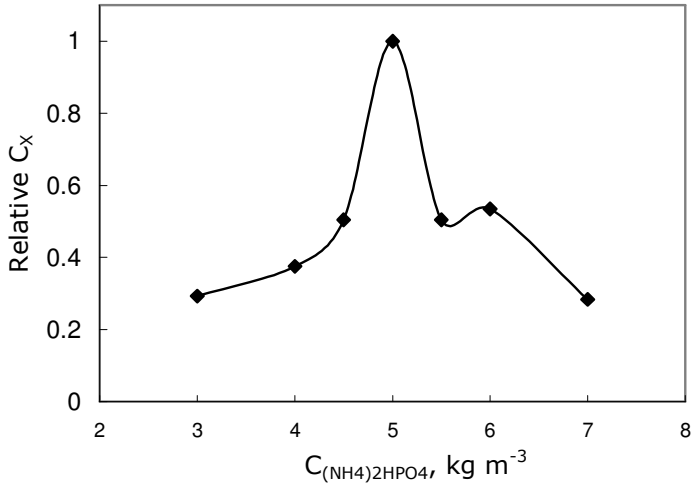


Figure 4.2 Variation of relative cell concentration with respect to $(NH_4)_2HPO_4$ concentration at t=17 h of the bioprocess

Table 4.2 Effect of the acid used to adjust initial pH of the production medium

Acid	C_X (kg m^{-3}) at t=17. h	Relative C_X wrt. H_2SO_4
H_2SO_4	0.28	1.0
H_3PO_4	0.30	1.1
HCl	0.24	0.85

4.1.5 Effect of Salt Solution on growth of the bacteria

Effect of the addition of salts was investigated by using the salt solution used in beta lactamase production (Çelik, and Çalık, 2004). Salt solution contained (kg m^{-3}): 250, $MgSO_4.7H_2O$; 1 , $FeSO_4.7H_2O$; 1, $ZnSO_4.7H_2O$; 7.5 $\times 10^{-2}$ $MnSO_4.H_2O$; 10^{-2}, $CuSO_4.5H_2O$. With the 33 µl addition of salt solution the

cell concentration increased to C_X =0.60 kg m^{-3} from 0.30 kg m^{-3} . The result show that the addition of inorganic ions to the medium is very vital for growth of the bacteria. New medium was named as glucose based defined (GBD) medium and contained (kg m^{-3}): glucose, 8; $(NH_4)_2HPO_4$, 5; $CaCl_2$, 0.2; KH_2PO_4, 2; $NaH_2PO_4.2H_2O$, 7.318; Na_2HPO_4, 0.0438; $Mg(CH_3COO)_2.4H_2O$, 87×10^{-3} ; 0.25 , $MgSO_4.7H_2O$; 10^{-3}, $FeSO_4.7H_2O$; 10^{-3}, $ZnSO_4.7H_2O$; 7.5×10^{-5} $MnSO_4.H_2O$; 10^{-5}, $CuSO_4.5H_2O$ with pH_0 =5, adjusted by H_3PO_4 .

4.1.6 Effect of Initial Glucose Concentration

By using the GBD medium without glucose, effect of the initial glucose concentrations was investigated at C_G^o =4, 6, 8, 10, and 16 kg m^{-3}. Between C_G^o =4-16 kg m^{-3} the cell concentration was not affected considerably. However, C_G =8 kg m^{-3} was chosen (Figure 4.3) .

4.1.7 Effects of Alternative Carbon Sources on Growth

The variations in cell concentration utilizing different carbon sources, i.e., glucose, fructose, citric acid, sucrose and maltose, were investigated, in GBD medium without glucose. The initial concentrations of the carbon sources were (kg m^{-3}): glucose, 8.0; fructose, 8.0; sucrose, 6.6; glycerol, 8.1; citric acid, 9.2; maltose, 7.98; adjusted such that in each case, the amount of carbon in the medium was equivalent to the amount of carbon in the medium containing 8.0 kg m^{-3} of glucose.

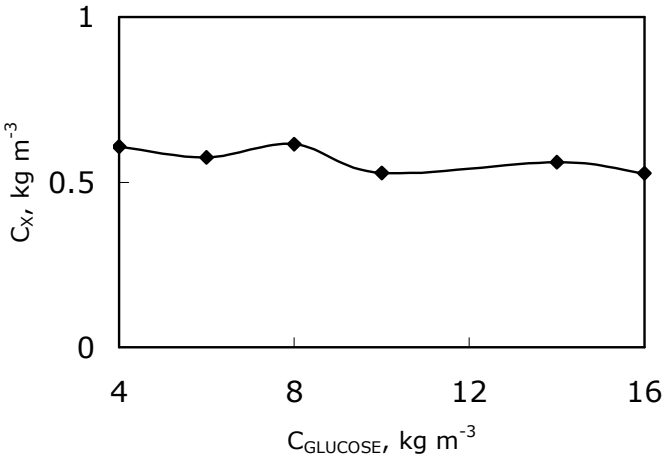


Figure 4.3 Effect of glucose concentration on biomass concentration

Although glucose and fructose are the isomers of each other and both of them enter to the carbon metabolism from the glycolysis pathway, use of fructose as the carbon source resulted 15 % increase in cell concentration. The inclination of this bacteria towards fructose- which exists at high amounts in fruit juices- has been supported by the the existence of this species in the spoiled fruit juices as a causative organism of off-flavor (Jensen, 1999; Pettipher et al. 1997; Splittstoesser et al., 1994; Yamazaki et al., 1998). On the other hand, citric acid entering the carbon metabolism from TCA cycle did not enhance cell generation probably because of the oxygen limitation, as the driving force in the TCA cycle is oxygen. The inability of this bacteria to utilize citric acid is consistent with literature (Darland and Brock, 1971). The cells can utilize sucrose as a carbon source if invertase is secreted to the extracellular medium which degrades sucrose into glucose and fructose, which are then transported into the cell via group translocation and enter the glycolysis pathway. On the other hand, sucrose may be transferred to the cell by group translocation and then hydrolysed in the presence of invertase into glucose and fructose. As seen in Figure 4.4, the cells were able to utilize sucrose; however, it was not preferred over fructose . Maltose, consisting of 2 glucose, is harder to hydrolyze than sucrose (Bailey and Ollis, 1986). Thus it

causes more ATP usage to the bacteria during being hydrolyzed. Therefore, the cell concentration was lower compared to the one obtained with sucrose.

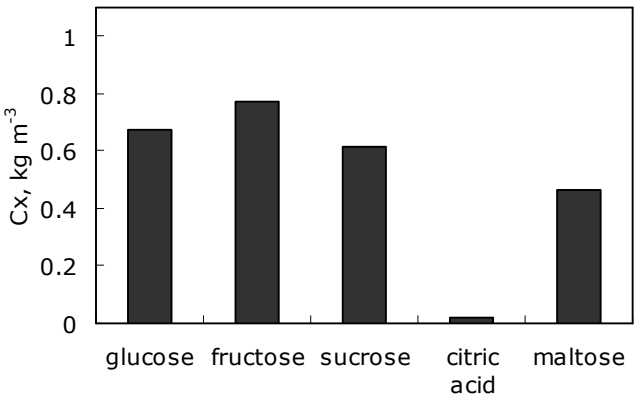


Figure 4.4 Effects of Different Carbon Sources on Biomass Concentration at t=17 h of the Bioprocess

4.1.8 Effect of Initial Fructose Concentration

Effect of initial fructose concentration was investigated at C_F^o =6, 8, 10, 15, 20, 30 kg m^{-3}. At C_F^o =6-15 kg m^{-3} cell concentration was not affected; however, higher initial fructose concentrations inhibited cell formation. At C_F^o = 20 and 30 kg m^{-3} , there were nearly no cell growth because of the substrate inhibition. C_F^o =8 kg m^{-3} was chosen, although higher or lower concentrations did not bring significant differences on biomass concentration (Figure 4.5). The medium containing (kg m^{-3}): fructose, 8; $(NH_4)_2HPO_4$, 5; $CaCl_2$, 0.2; KH_2PO_4, 2; $NaH_2PO_4.2H_2O$, 7.318; Na_2HPO_4, 0.0438; $Mg(CH_3COO)_2.4H_2O$, 87×10^{-3} ; 0.25 , $MgSO_4.7H_2O$; 10^{-3}, $FeSO_4.7H_2O$; 10^{-3}, $ZnSO_4.7H_2O$; 7.5 $\times10^{-5}$ $MnSO_4.H_2O$; 10^{-5} , $CuSO_4.5H_2O$ with pH_0 =5, adjusted by H_3PO_4 was used at further medium design experiments. This medium was called as fructose based defined medium (FDM).

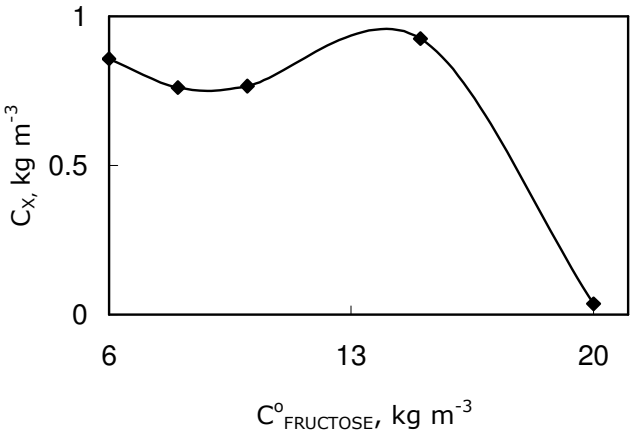


Figure 4.5 Effect of fructose concentration on cell concentration

4.1.9 Effect of Salt Solution Concentration

The use of salt solution with the carbon source glucose increased cell concentration; therefore, effect of the salt solution was investigated in fructose based defined medium. The highest cell concentration was obtained respectively as C_X=0.92 kg m^{-3} (Table 4.3) in the medium containing (kg m^{-3}): fructose, 8; $(NH_4)_2HPO_4$, 5; $CaCl_2$, 0.2; KH_2PO_4, 2; $NaH_2PO_4.2H_2O$, 7.318; Na_2HPO_4, 0.0438; $Mg(CH_3COO)_2.4H_2O$, 87×10^{-3} ; 0.5 , $MgSO_4.7H_2O$; 2×10^{-3}, $FeSO_4.7H_2O$; 2×10^{-3}, $ZnSO_4.7H_2O$; 15 $\times10^{-}$ $MnSO_4.H_2O$; 2×10^{-5}, $CuSO_4.5H_2O$ with pH_0 =5, adjusted by H_3PO_4

Table 4.3 Effect of the salt concentration on cell concentration

Concentrations of Salts (kg m^{-3}) in addition to $CaCl_2$, 0.2; KH_2PO_4, 2; $NaH_2PO_4.2H_2O$, 7.318; Na_2HPO_4, 0.0438; $Mg(CH_3COO)_2.4H_2O$, 87×10^{-3} .	Cx, kg m^{-3}
$C_{MgSO4.7H2O}=0.25$,$C_{FeSO4.7H2O}=10^{-3}$,$C_{ZnSO4.7H2O}=10^{-3}$ $C_{MnSO4.H2O}=7.5\times10^{-5}$, $C_{CuSO4.5H2O}=10^{-5}$	0.76
$C_{MgSO4.7H2O}=0.5$ $C_{FeSO4.7H2O}=2\times10^{-3}$ $C_{ZnSO4.7H2O}=2\times10^{-3}$ $C_{MnSO4.H2O}=15\times10^{-5}$ $C_{CuSO4.5H2O}=2\times10^{-5}$	0.92
$C_{MgSO4.7H2O}=0.75$ $C_{FeSO4.7H2O}=3\times10^{-3}$ $C_{ZnSO4.7H2O}=3\times10^{-3}$ $C_{MnSO4.H2O}=22.5\times10^{-5}$ $C_{CuSO4.5H2O}=3\times10^{-5}$	0.0193
$C_{MgSO4.7H2O}=1$ $C_{FeSO4.7H2O}=4\times10^{-3}$ $C_{ZnSO4.7H2O}=4\times10^{-3}$ $C_{MnSO4.H2O}=30\times10^{-5}$ $C_{CuSO4.5H2O}=4\times10^{-5}$	0.0180
$C_{MgSO4.7H2O}=1.25$ $C_{FeSO4.7H2O}=5\times10^{-3}$ $C_{ZnSO4.7H2O}=5\times10^{-3}$ $C_{MnSO4.H2O}=37.5\times10^{-5}$ $C_{CuSO4.5H2O}=5\times10^{-5}$	0.0193

4.1.10 Effect of $Mg(CH_3COO)_2.4H_2O$ Concentration

The effect of $Mg(CH_3COO)_2.4H_2O$ concentration was investigated at $C_{Mg(CH3COO)2.4H2O}$= 0, 0.087, 0.174, 0.239 kg m^{-3}. $Mg(CH_3COO)_2.4H_2O$ concentration was selected to be 0.087 kg m^{-3} (Figure 4.6) .

4.1.11 Effect of $MgSO_4.7H_2O$ Concentration

Effect of supplementing extra $MgSO_4.7H_2O$ into the previous medium was investigated. The cell concentration was not so much influenced by the addition of $MgSO_4.7H_2O$ (Figure 4.7) . However $C^{o}_{MgSO4.7H2O}$ = 1.0 kg m^{-3} was chosen . Thus the medium became the following (kg m^{-3}): fructose, 8; $(NH_4)_2HPO_4$, 5; $CaCl_2$, 0.2; KH_2PO_4, 2; $NaH_2PO_4.2H_2O$, 7.318; Na_2HPO_4, 0.0438; $Mg(CH_3COO)_2.4H_2O$, 87×10^{-3} ; 1.0 , $MgSO_4.7H_2O$; 2×10^{-3}, $FeSO_4.7H_2O$; 2×10^{-3}, $ZnSO_4.7H_2O$; 15 $\times10^{-5}$ $MnSO_4.H_2O$; 2×10^{-5}, $CuSO_4.5H_2O$-with pH_0 =5, adjusted by H_3PO_4.

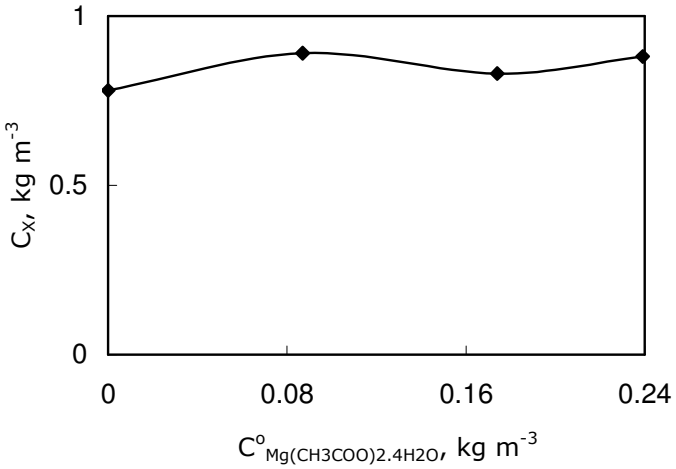


Figure 4.6 Effect of $Mg(CH_3COO)_2.4H_2O$ concentration on cell concentration

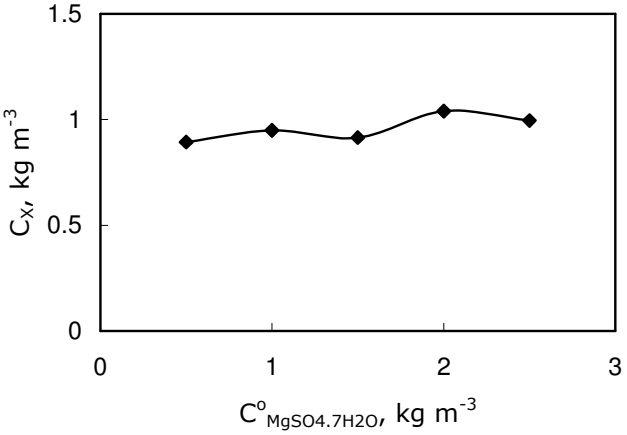


Figure 4.7 Variation of cell concentration with $MgSO_4.7H_2O$ concentration

4.1.12 Final Medium

Optimum medium for cell growth contains the following initial medium (kg m^{-3}): fructose, 8; $(NH_4)_2HPO_4$, 5; $CaCl_2$, 0.2; KH_2PO_4, 2; $NaH_2PO_4.2H_2O$, 7.318; Na_2HPO_4, 0.0438; $Mg(CH_3COO)_2.4H_2O$, 87×10^{-3} ; 1 , $MgSO_4.7H_2O$;

2×10^{-3}, $FeSO_4.7H_2O$; 2×10^{-3}, $ZnSO_4.7H_2O$; 15×10^{-5}, $MnSO_4.H_2O$; 2×10^{-5}, $CuSO_4.5H_2O$ [with pH_0 =5, adjusted by H_3PO_4 . With this optimum medium, biomass concentration at t=17 h of the bioprocess was 0.89 kg m^{-3} while at the very begining it was 0.28 kg m^{-3}.

4.2 Bioreactor Operation Parameters

4.2.1 Effects of initial pH and Temperature

Effects of temperature at T= 50, 55, 60 °C and initial pH at pH_o= 3, 3.5, 4, 4.5, 5, 5.5 were investigated in shake-bioreactors. For 50 & 55 °C, optimum initial pH for Cx was 5 while for 60 °C it decreased to 4.5 (Figure 4.8). The highest cell concentration was obtained at 55 °C and initial pH of 5.

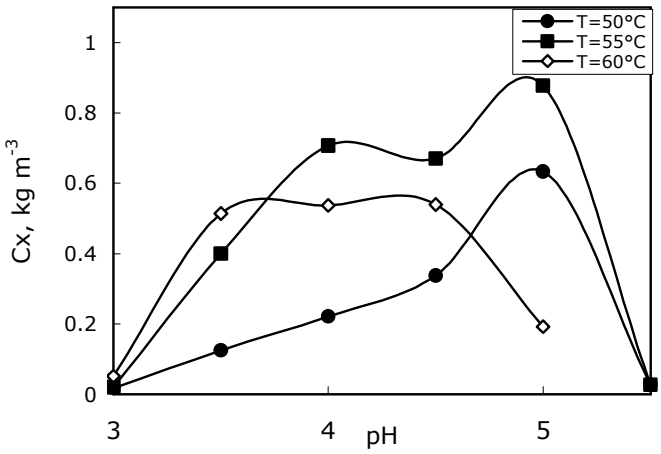


Figure 4.8 Effects of different production temperatures and initial pHs of the medium on biomass concentrations at t=17 h of the bioprocess

4.3 Product, By-product Distributions and Yield Values in the Designed Defined Medium

As it is seen in Figure 4.9, in shake-bioreactor experiments L-tryptophan (Trp) production was growth associated and the highest Trp concentration was obtained as 0.204 kg m^{-3} at t= 17.5 h of the bioprocess. In addition to Trp the concentration of the other amino acids were also determined and given in Table 4.4. Besides Trp, phenylalanine at a maximum concentration of 0.0106 kg m^{-3} was obtained at t= 40 h of the bioprocess. However there was no Tyr production throughout the bioprocess.

At t=0 h, Asn, Asp, Glu, Tyr, Cys, Phe and Trp were exist in the medium, coming from the precultivation medium. However, after t>0, only the amino acids namely Asn, Ser, Cys, Phe and Trp were found in the fermentation broth (Table 4.4). Asp, Glu, Gly and Tyr-which were available at t=0 h, were transported due to the demand of the cell. On the other hand, His, Arg, Thr, Ala, Pro, Val, Iso+Leu were not observed in the fermentation broth throughout the bioprocess. Total concentration of the aminoacids increased from t=0 to t=17.5 h , reaching a maximum value of 0.24 kg m^{-3} at t=17.5 h of the bioprocess. After t=17.5 h, total amino acid concentration decreased with a lower rate than the rate of increase of total amino acid concentration in the period of t=0-17.5 h.

Extracellular organic acid profiles of the shake-bioreactor fermentation are given in Table 4.5. The only organic acid existing in the extracellular medium at t=0 h was acetic acid-coming from the precultivation medium. The concentration of acetic acid slightly increased until t= 17.5 h of the process and then decreased. Besides acetic acid, succinic acid, α-ketoglutarate, lactic acid, butyric acid and gluconic acid were also available in the fermentation broth (Table 4.5). Among these organic acids, succinic acid and α-ketoglutarate are the ones produced in TCA cycle. The excretion of these TCA organic acids to the fermentation broth may be the sign of inefficient amount of oxygen or electron transport to transform these organic acids into the other TCA-products. Namely, if the driving force of oxygen had been higher, these products would not have been in the fermentation broth. The organic acid that had the highest concentration was butyric acid-1.83 kg m^{-3} at

t=26.5 h of the bioprocess. Concentration of the total organic acids (ΣC_{OA}) increased in the period of t=0-26.5 h of the bioprocess. After reaching the maxima at t=26.5 h, ΣC_{OA} decreased slightly. Butyric acid only existed at shake-bioreactor experiment, not at any of the bioreactor experiments.

The variations in specific growth rate and yield coefficients in the fermentation broth with cultivation time in shake-bioreactor are given in Table 4.6. μ_{max} was 0.195 at t=4.5 h of the bioprocess. q_S , q_P , μ decreased in t=4.5-17.5 h of the bioprocess. Maximum $Y_{X/S}$, $Y_{P/S}$ and $Y_{P/X}$ were obtained at t=14 h of the bioprocess. The maintenance coefficient was found to be 0.0067 kg kg^{-1} h^{-1} for t=0-7 h of the bioprocess. Leudeking-Piret constants were found to be the following: α 0.238 (g Trp/(g cell.hour)) , β -0.00228 (g Trp/g cell).

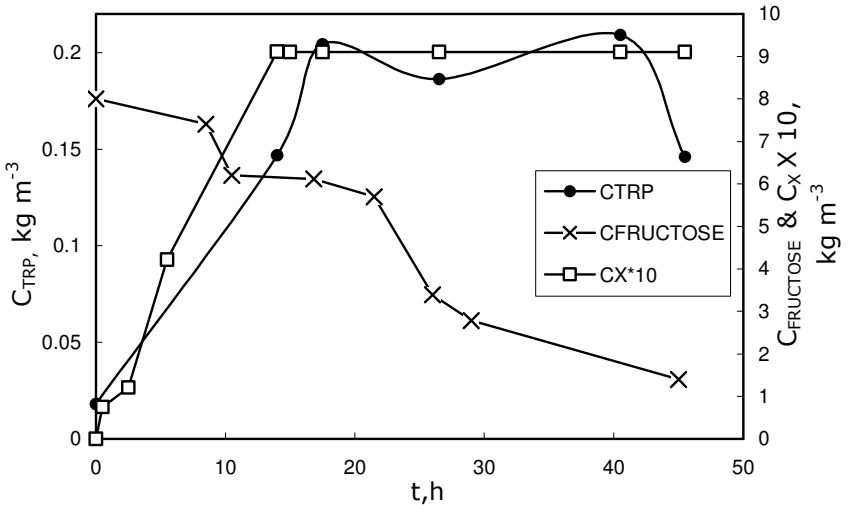


Figure 4.9 Variation of fructose, cell, L-tryptophan concentrations with the cultivation time at N=175 min^{-1}, T=55°C, pHo=5

Table 4.4 The variations in amino acid concentrations in the fermentation broth with cultivation time, T=55°C, N=175 min^{-1} in shake bioreactor

Cultivation time, h						
C_{AA}, kg m^{-3}	t=0	t=14	t=17.5	t=26.5	t=40.5	t=45.5
Asn	0	0.011	0.015	0.0104	0.0087	0.0066
Asp	0.030	0	0	0	0	0
Glu	0.0017	0	0	0	0	0
Gly	0.0034	0	0	0	0	0
Ser	0.012	0	0.0067	0.011	0.0048	0
His	0	0	0	0	0	0
Arg	0	0	0	0	0	0
Thr	0	0	0	0	0	0
Ala	0	0	0	0	0	0
Pro	0	0	0	0	0	0
Tyr	0	0	0	0	0	0
Val	0.0036	0	0	0	0	0
Cys	0	0	0.006	0.0044	0.0037	0.0076
Iso+Leu	0.011	0	0	0	0	0
Phe	0	0.008	0.009	0.0086	0.01	0.0086
Trp	0.006	0.147	0.204	0.186	0.209	0.146
ΣC_{AA}, kg m^{-3}	0.018	0.166	0.241	0.22	0.237	0.169

Table 4.5 The variations in organic acid concentrations in the fermentation broth with cultivation time, T=55°C, N=175 min^{-1} in shake-bioreactor

Cultivation time, h					
C_{OA}, kg m^{-3}	t=0	t=14	t=17.5	t=26.5	t=40.5
Cit	0	0	0	0	0
α-kG	0	0	0	0	1.47
Suc	0	0.317	0.287	0	0
Pru	0	0	0	0	0
Ac	0.13	0.09	0.095	0.022	0
Lac	0	0	0	0	0.091
But	0	0	0	1.83	0
Glu	0	0	0	0	0
Gluc	0	0.007	0.007	0	0
ΣC_{OA} , kg m^{-3}	0.13	0.414	0.389	1.85	1.56

Table 4.6 The variations in specific growth rate and yield coefficients in the fermentation broth with cultivation time, T=55°C, N=175 min^{-1} in shake-bioreactor

t	μ	qs	q_P	$Y_{X/S}$	$Y_{P/S}$	$Y_{P/X}$
(h)	(h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})
4.5	0.19	0.22	0.029	0.88	0.13	0.15
7	0.13	0.15	0.019	0.88	0.13	0.15
14	0.03	0.015	0.014	2.33	0.95	0.41
17.5	0	0.098	0.008	0	0.080	-
26.5	0	0.33	-	0	-	-
40.5	0	0.095	-	0	-	-
Overall				0.15	0.03	0.21

4.4 Bioreactor Experiments

Using the optimum medium with *Bacillus acidocaldarius* NRRC-207 F Rev.2/78, the fermentation and oxygen transfer characteristics of the bioprocess were investigated in the 3.0 dm^3 pilot scale batch bioreactor (Braun CT2-2) where V_R =1.65 dm^3. Throughout the bioprocess cell, fructose, amino acid and organic acid concentrations, dissolved oxygen, pH, oxygen uptake rate, overall mass transfer coefficient, specific growth rate, yield and maintenance coefficients were determined. In the pilot scale bioreactor, the effects of oxygen transfer were investigated at four different conditions at the parameters air inlet rates of Q_O/V_R =0.2, and 0.5 vvm, and agitation rates of N= 250, 500, 750 min^{-1}.

The effect of pH on aromatic amino acid production capacity was investigated at pH=5 uncontrolled and controlled operations as optimum initial pH for cell growth was found to be 5 at shake-bioreactor. Controlled-pH experiments were carried out with 5M KOH and 5 M H_3PO_4. The abbreviation of the bioreactor operation conditions applied is given in Table 4.7.

Table 4.7 Oxygen transfer conditions and pH conditions and their abbreviations

Oxygen Transfer Condition Applied N, min^{-1} , Q_O/V_R, vvm	pH strategy	Abbreviation
N = 250, Q_O/V_R = 0.2	pH_C=5.0	LimOT-pH_C
N = 250, Q_O/V_R = 0.5	pH_C=5.0	LOT -pH_C
N = 250, Q_O/V_R = 0.5	pH_{UC}=5.0	LOT –pH_{UC}
N = 500, Q_O/V_R = 0.5	pH_C=5.0	MOT-pH_C
N = 500, Q_O/V_R = 0.5	pH_{UC}=5.0	MOT-pH_{UC}
N = 750, Q_O/V_R = 0.5	pH_{UC}=5.0	HOT –pH_{UC}

4.4.1 pH Profiles

The variations in pH with cultivation time, air inlet rate, agitation rate and pH strategy are given in Figure 4.10. In all uncontrolled-pH conditions, N = 250 min^{-1}, Q_O/V_R = 0.5 vvm (LOT-pH_{UC}) ;N = 500 min^{-1}, Q_O/V_R = 0.5 vvm (MOT -pH_{UC}); N = 750 min^{-1}, Q_O/V_R = 0.5vvm (HOT-pH_{UC}), pH decreased considerably at t=0-17 h. At LOT-pH_{UC} pH decreased throughout the process and at the end of the bioprocess its value was pH=3.0. However at MOT -pH_{UC} and HOT-pH_{UC}, pH of the medium decreased from pH=5 to pH=3.5 at t=0-17 h and t=0-15 h of the bioprocesses, respectively; and then reached to the stationary phase.

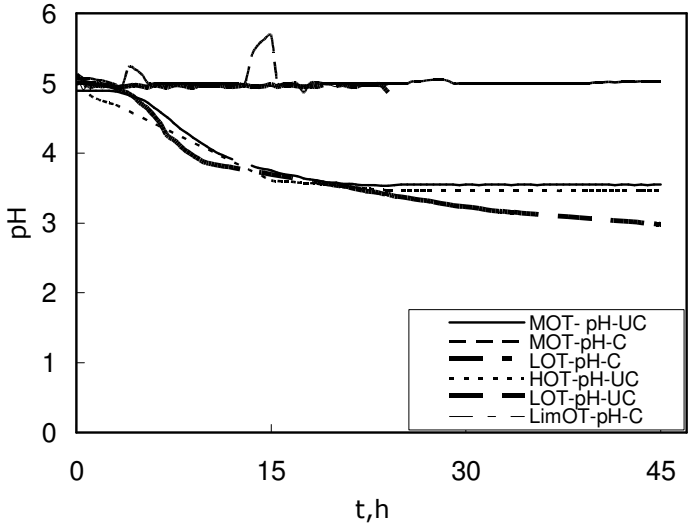


Figure 4.10 The variations in pH with the cultivation time, air inlet rate, agitation rate and pH strategy

4.4.2 Dissolved Oxygen Profiles

The variations in dissolved oxygen concentration with cultivation time, air inlet rate, agitation rate and pH strategy are given in Figure 4.11. C_{DO} depends on the extent of the oxygen transfer rate (OTR) to the medium and the oxygen uptake rate (OUR) of the cells. At 55 °C, solubility of oxygen in water at standard pressure, C^*_O , is 0.175 mol m^{-3} , based on 'International Critical Tables' (Rychtera, 2002). Among controlled-pH operations that are LimOT-pH_C , LOT -pH_C from t= 2.5 h and t=4.5 h of the bioprocesses respectively, there was no oxygen accumulation; thus, OTR was equal to the OUR= OTR. At LimOT-pH_C case, the DO was depleted at a higher rate than that of LOT –pH_C.

Among uncontrolled-pH operations, at LOT –pH_{UC}, DO in the medium was completely depleted within the first 4.5 hours. However at MOT-pH_{UC}, the C_{DO} decreased in the first 20 hours; after t= 20 h, due to the decrease in oxygen uptake rate C_{DO} increased. At HOT –pH_{UC}, C_{DO} continuously decreased throughout the bioprocess at a low rate and the lowest C_{DO} was 0.14 mol/m^3 at the end of the bioprocess.

Dissolved oxygen profiles of LOT–pH_{UC} and LOT–pH_C were alike. On the other hand, the locus of the C_{DO} vs. t profiles obtained for at MOT–pH_{UC} and MOT–pH_C conditions were similar; however controlled operations resulted with lower C_{DO} values and higher decrease rates at t=0-15 h of the bioprocess due to higher metabolic activity.

4.4.3 Fructose and Cell Concentration Profiles

The variations in fructose concentration with the cultivation time, air inlet rate, agitation rate and pH strategy are given in Figure 4.12. Among the controlled-pH conditions (LimOT –pH_C, LOT –pH_C, MOT-pH_C), the highest and the lowest fructose consumptions were obtained respectively at MOT-pH_C

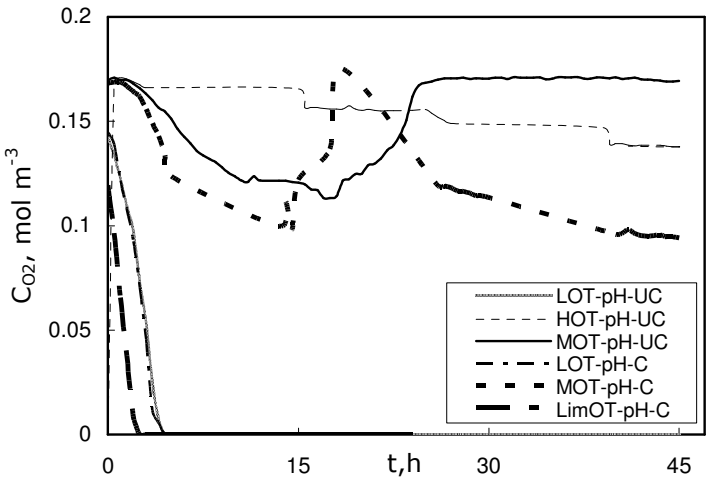


Figure 4.11 The variations in dissolved oxygen concentration with the cultivation time, air inlet rate, agitation rate and pH strategy

(t= 24 h) and at LimOT-pH_C (t=24 h) indicating that higher amount of oxygen the medium had, the more fructose the bacteria used.

At uncontrolled-pH operations, fructose consumption decreased with the increasing agitation rate. The highest fructose consumption occurred at LOT – pH_{UC} ; whereas the lowest fructose consumption rate was obtained at HOT – pH_{UC} .

Higher fructose consumption rates were obtained at controlled-pH operations compared to the uncontrolled-pH operations indicating the effective operation of intracellular reaction network at pH=5.0.

The variations in cell concentrations with the cultivation time, air inlet rate, agitation rate and pH strategy are given in Figure 4.13. In general, cell

concentration started to increase at t= 0 h of the fermentations, and reached to the stationary phase at t=15 h.

Among the controlled-pH conditions, the highest and the lowest cell concentrations were attained as C_X=1.59 kg m^{-3} at MOT-pH_C, and as C_X=0.37 kg m^{-3} at LimOT-pH_C, respectively. At the pH-controlled operations, cell concentration in the medium increased as the amount of oxygen in the medium increased.

The highest cell concentration was obtained as C_X=1.59 kg m^{-3} at MOT-pH_C, which had also the highest fructose consumption rate. Contrariwise, the lowest cell concentration (C_X=0.37 kg m^{-3}) and fructose consumption rate were obtained at LimOT-pH_C. Under uncontrolled-pH operations, pH and the cell concentration profiles were interrelated, as with the decrease in pH cell concentration increased. When pH reached to the stationary phase, cell growth was also reached to the stationary phase. In contrast to controlled-pH operations, at uncontrolled-pH operations, the cell concentration and fructose consumption decreased with the increase in agitation rate. The highest and the lowest cell concentrations were obtained as C_X= 0.896 kg m^{-3} and C_X= 0.598 kg m^{-3}, respectively at LOT–pH_{UC} at t=45 h and at HOT–pH_{UC} at t=15 h of the bioprocesses. At LOT –pH_{UC} the cell concentration increased throughout the bioprocess (t=0-45 h); and the continuous increase in cell concentration was in harmony with the continuous decrease in pH. In addition to these, the highest and the lowest fructose consumptions were also occurred at LOT –pH_{UC} and at HOT–pH_{UC}.

Overall cell yields on substrate ($Y'_{X/S}$) obtained at the bioreactor operations conditions investigated are given in Table 4.8 and 4.9. At controlled-pH operations, the highest $Y'_{X/S}$was achieved at MOT-pH_C while the lowest $Y'_{X/S}$ was obtained at LOT-pH_C in t=0-24 h period (Table 4.8). At uncontrolled-pH operations, the highest and the lowest $Y'_{X/S}$ values were attained at HOT –pH_{UC} and LOT –pH_{UC} in t=0-24 h period, respectively (Table 4.9).

At MOT condition, controlled-pH operations gave a higher $Y'_{X/S}$ than the uncontrolled-pH operations. At LOT condition, there was not a significant difference between the $Y'_{X/S}$ values of both controlled-pH and uncontrolled-pH operations due to the oxygen limitation.

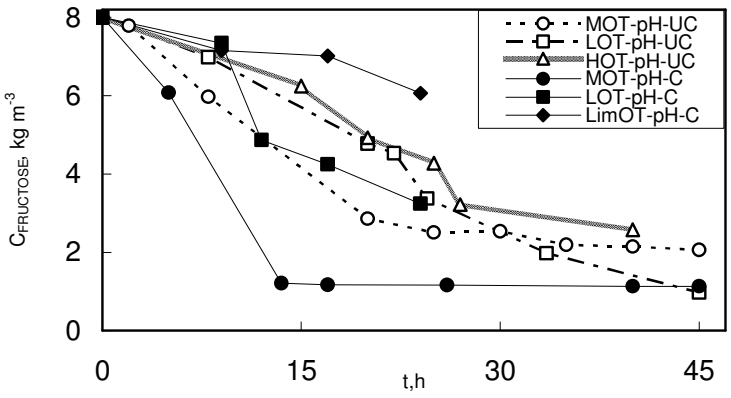


Figure 4.12 The variations in fructose concentrations with the cultivation time, air inlet rate, agitation rate and pH strategy

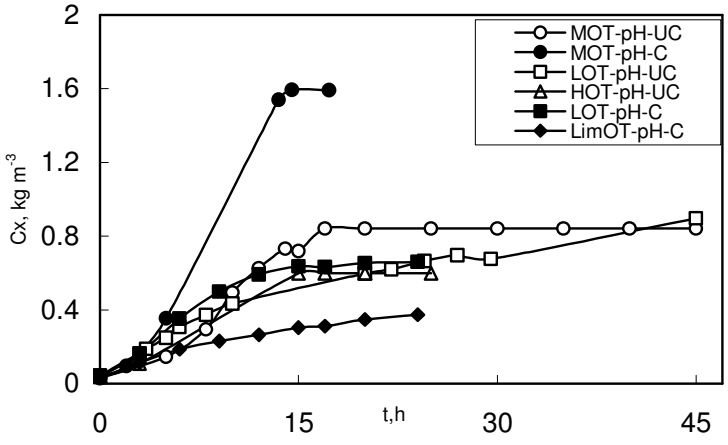


Figure 4.13 The variations in cell concentrations with the cultivation time, air inlet rate, agitation rate and pH strategy

Table 4.8 Overall Yields and maximum cell concentrations at pH-controlled cases

Bioreactor Operation Conditions	The highest C_X (kg m^{-3}) and its time	$Y_{X/S}'$ (t=0-24 h)
MOT-pH_C	1.59 at t=14.5 h	0.23
LOT-pH_C	0.66 at t=24 h	0.133
LimOT-pH_C	0.37 at t=24 h	0.18

Table 4.9 Overall Yields and maximum cell concentrations at pH-uncontrolled cases

Bioreactor Operation Condition	The highest C_X (kg m^{-3}) and its time	$Y_{X/S}'$ (t=0-24 h ; t= 0-40 h)
LOT –pH_{UC}	0.90 at t=45 h	0.14 at t=0-24 h 0.12 at t=0-40 h
MOT –pH_{UC}	0.84 at t=17 h	0.15 at t=0-24 h 0.14 at t=0-40 h
HOT –pH_{UC}	0.60 at t=15 h	0.16 at t=0-24 h 0.11 at t=0-40 h

4.4.4 Tryptophan Concentration Profiles

The variations in Tryptophan (Trp) concentrations with cultivation time, air inlet rate, agitation rate and pH strategy are given in Figure 4.14. Among all the investigated bioreactor operation conditions, the highest Trp production was attained at LimOT-pH_C condition as 0.32 kg m^{-3} at t=17 h of the bioprocess while the lowest Trp production was obtained at MOT-pH_{UC} as 0.13 kg m^{-3} at t=45 h of the bioprocess.Under the controlled-pH operations

with the increase in agitation/aeration rate Trp production decreased while fructose consumption and cell formation increased. Thus, with the increase in oxygen, carbon directed to biomass synthesis pathway, rather than aromatic amino acid synthesis pathway.

Among uncontrolled-pH operations, at only LOT-pH_{UC} , maximum Trp production, maximum fructose consumption and maximum cell concentration concepts were realized simultaneously and the highest Trp was obtained as 0.224 kg m^{-3} at t= 10 h of the bioprocess. At HOT-pH_{UC} similar to LOT-pH_{UC}, 0.224 kg m^{-3} Trp was produced but with a 35 h shift in the cultivation time. At both MOT and LOT conditions, pH-control produced higher amounts of Trp than the uncontrolled conditions.

Overall product yield on substrate ($Y'_{P/S}$) and the product generated per cell formed ($Y'_{P/X}$) in the period of t=0-t_{max} , where t_{max} corresponds to the time of maximum Trp concentration are given in Table 4.10 and 4.11. Highest $Y'_{P/S}$, 0.298 kg kg^{-1}, was obtained at LimOT-pH_C. The yield obtained with the wild type *Bacillus* acidocaldarius at LimOT-pH_C was much higher than that of Dodge and Gernster, reporting a 0.176 kg kg^{-1} Trp yield on glucose with a mutant *E.coli* producing 42.3 kg m^{-3} Trp (2002). At controlled operations, as lower oxygen existed in the medium, higher $Y'_{P/S}$ values were attained. At MOT condition, pH control gave a lower $Y'_{P/X}$ as at MOT-pH_C higher amounts of carbon was directed to the cell generation ,while at LOT condition pH control gave a higher $Y'_{P/X}$ as higher Trp and lower Cx was attained at controlled-pH operation of LOT.

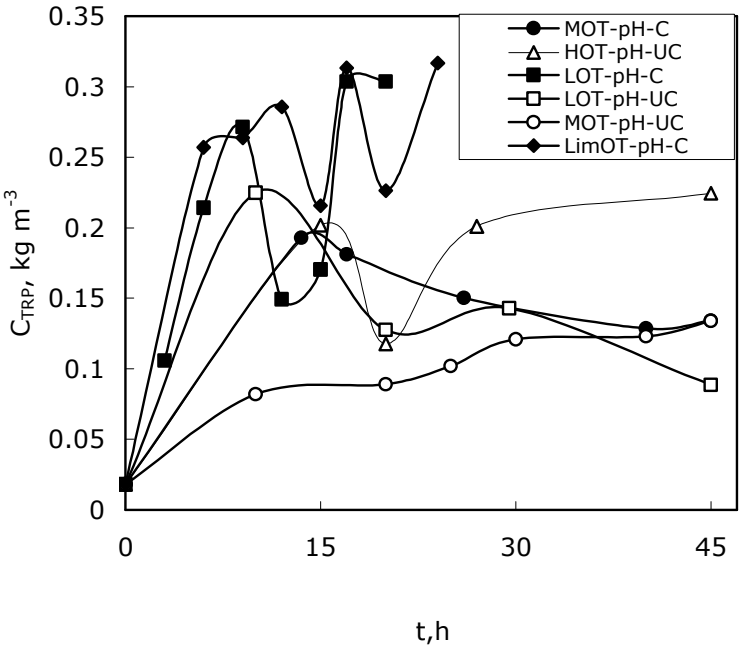


Figure 4.14 The variations in tryptophan concentrations with the cultivation time, air inlet rate, agitation rate and pH strategy

Table 4.10 Overall Trp Yields at controlled-pH conditions

Bioreactor Operation Condition	Maximum Trp Concentration (kg m^{-3}) and its time, t_{MAX}	Overall Yield of Trp on substrate (kg/kg) in t=0-t_{MAX}	Overall Yield of Trp on cell (kg/kg) in t=0-t_{MAX}
LimOT-pH_C	0.313 , 17	0.298	1.04
LOT-pH_C	0.3036, 17	0.076	0.474
MOT-pH_C	0.193, 13.5	0.026	0.116

Table 4.11 Overall Trp Yields at uncontrolled-pH conditions

Bioreactor Operation Condition	Maximum Trp Concentration (kg m^{-3}) and its time, t_{MAX}	Overall Yield of Trp on substrate (kg/kg) in t=0-t_{MAX}	Overall Yield of Trp on cell (kg/kg) in t=0-t_{MAX}
LOT-pH_{UC}	0.225, 10	0.149	0.365
MOT-pH_{UC}	0.134, 45	0.019	0.143
HOT-pH_{UC}	0.224, 45	0.038	0.363

4.4.5 Extracellular Amino Acid Profiles

The variations in extracellular amino acid concentrations with cultivation time, air inlet rate, agitation rate and pH strategy are given in Tables 4.12, 4.13, 4.14, 4.15, 4.16, 4.17. At t=0 h, for all bioreactor conditions, the following amino acids that are coming from the precultivation were present in the fermentation broth: Asn, Asp, Glu, Gly, Tyr, Cys, Phe, Trp; medium. At all controlled-pH operations, Arg, Thr, Ala, Pro, Tyr, Val were not detected in the fermentation broths; whereas His, Cys; Glu; Asp, Gln, Ser were excreted into the fermentation broth only at MOT-pH_C; LOT-pH_C and LimOT-pH_C, respectively. At the cultivation time that corresponds to the time in which the highest Trp was obtained, the highest ΣC_{AA} was attained at LimOT-pH_C as 0.36 kg m^{-3} at t=24 h and the lowest ΣC_{AA} was obtained at MOT-pH_C as 0.22 kg m^{-3} at t=17 h. As the oxygen in the medium increased, obtained maximum ΣC_{AA} decreased.

In all uncontrolled-pH operations, Asp coming from the precultivation was depleted after t=0 h. At all uncontrolled-pH conditions Asp, Gln, His, Arg, Thr, Ala, Pro, Val were not detected in the medium at all during the bioprocesses. Glu, Tyr and Iso+Leu were exist at MOT-pH_{UC}. At LOT-pH_{UC} , maximum ΣC_{AA} was 0.25 kg m^{-3} at t=10 h. At MOT-pH_{UC} , maximum ΣC_{AA} was 0.154 kg m^{-3} at t=45 h. At HOT-pH_{UC} , maximum ΣC_{AA} was 0.27 kg m^{-3} at t=45 h. Maximum ΣC_{AA} was at HOT-pH_{UC}. pH control increased the amount of the total amino acids excreted into the fermentation broth.

It is apparent that this bacteria mostly produces Trp. Trp is the amino acid that is produced at the highest concentration, with the highest frequency. Phe production is at a lower amount and frequency than Trp production. Tyr production is very rare, among all the aromatic amino acids produced. Phe and Tyr were not produced as much as Trp. Tyr was produced only at MOT-pH_C condition, with a maximum concentration of 0.002 kg m^{-3} at t=45 h of the bioprocess. However, Phe was produced at all conditions and at a higher concentration than Tyr. Among all the bioreactor experiment, maximum Phe concentration was observed as 0.018 kg m^{-3} at t=40, at MOT-pH_C condition; while minimum Phe concentration was observed as 0.0033 and 0.0032 kg m^{-3} at t=12, and t=3 h of LOT-pH_C and LimOT-pH_C conditions respectively. At MOT condition, more Phe was produced at uncontrolled-pH operation. However, at LOT condition more Phe was produced at controlled-pH operation.

Related with the aromatic amino acids, one can conclude that either the activity of the enzymes from the main branch point chorismate towards Tyr should be the lowest, and from chorismate towards Phe should be low, and from chorismate towards Trp should be the highest or there might be strong regulation towards Tyr and weak regulation towards Trp.

Table 4.12 The variations in amino acid concentrations in the fermentation broth with cultivation time, T=55°C, N=500 min^{-1}, Q_0/V_R=0.5 vvm, pHo=5 uncontrolled

Cultivation time, h							
C_{AA}, kg m^{-3}	t=0	t=10	t=20	t=25	t=30	t=40	t=45
Asn	0.030	0.031	0.0067	0.006	0.0098	0	0
Asp	0.0017	0	0	0	0	0	0
Glu	0.0034	0	0	0	0	0.0069	0.011
Gly	0.012	0.0071	0	0.0036	0.0026	0	0
Ser	0	0	0	0	0	0	0
His	0	0	0	0	0	0	0
Arg	0	0	0	0	0	0	0
Thr	0	0	0	0	0	0	0
Ala	0	0	0	0	0	0	0
Pro	0	0	0	0	0	0	0
Tyr	0.0036	0	0	0	0	0.0019	0.0023
Val	0	0	0	0	0	0	0
Cys	0.011	0.0078	0.0043	0.002	0	0	0
Iso+Leu	0	0	0	0	0	0.0026	0
Phe	0.006	0.0037	0.0040	0.004	0.0045	0.0076	0.0062
Trp	0.018	0.082	0.089	0.102	0.121	0.123	0.134
ΣC_{AA}, kg m^{-3}	0.086	0.132	0.104	0.119	0.138	0.142	0.154

Table 4.13 The variations in amino acid concentrations in the fermentation broth with cultivation time, T=55°C, N=250 min^{-1}, Q_0/V_R=0.5 vvm, pHo=5 uncontrolled

Cultivation time, h					
C_{AA}, kg m^{-3}	t=0	t=10	t=20	t=29.5	t=45
Asn	0.030	0.015	0.036	0.015	0.012
Asp	0.0017	0	0	0	0
Glu	0.0034	0	0	0	0
Gly	0.012	0	0.0084	0	0
Ser	0	0	0	0.006	0
His	0	0	0	0	0
Arg	0	0	0	0	0
Thr	0	0	0	0	0
Ala	0	0	0	0	0
Pro	0	0	0	0	0
Tyr	0.0036	0	0	0	0
Val	0	0	0	0	0
Cys	0.011	0	0	0.0045	0.005
Iso+Leu	0	0	0	0	0
Phe	0.006	0.012	0.011	0.010	0.0065
Trp	0.018	0.225	0.127	0.143	0.088
ΣC_{AA}, kg m^{-3}	0.086	0.252	0.184	0.179	0.113

Table 4.14 The variations in amino acid concentrations in the fermentation broth with cultivation time, T=55°C, N=750 min^{-1}, Q_0/V_R=0.5 vvm, pHo=5 uncontrolled

Cultivation time, h					
C_{AA}, kg m^{-3}	t=0	t=15	t=20	t=27	t=45
Asn	0.030	0.012	0.012	0.006	0.032
Asp	0.0017	0	0	0	0
Glu	0.0034	0	0	0	0
Gly	0.012	0	0	0	0
Ser	0	0	0.014	0.006	0
His	0	0	0	0	0
Arg	0	0	0	0	0
Thr	0	0	0	0	0
Ala	0	0	0	0	0
Pro	0	0	0	0	0
Tyr	0.0036	0	0	0	0
Val	0	0	0	0	0
Cys	0.011	0.005	0.0035	0	0.004
Iso+Leu	0	0	0	0	0
Phe	0.006	0.01	0.010	0.009	0.011
Trp	0.018	0.202	0.117	0.20	0.22
ΣC_{AA}, kg m^{-3}	0.086	0.228	0.157	0.22	0.272

Table 4.15 The variations in amino acid concentrations in the fermentation broth with cultivation time, T=55°C, N=500 min^{-1}, Q_0/V_R=0.5 vvm, pHo=5 controlled

Cultivation time						
C_{AA}, kg m^{-3}	t=0	t=13.5	t=17	t=26	t=40	t=45
Asn	0.030	0.012	0.021	0.017	0.017	0.016
Asp	0.0017	0	0	0	0	0
Glu	0.0034	0	0	0	0	0
Gly	0.012	0	0	0	0	0
Ser	0	0	0	0	0	0
His	0	0	0	0	0.003	0.0026
Arg	0	0	0	0	0	0
Thr	0	0	0	0	0	0
Ala	0	0	0	0	0	0
Pro	0	0	0	0	0	0
Tyr	0.0036	0	0	0	0	0
Val	0	0	0	0	0	0
Cys	0.011	0.004	0.004	0	0.005	0.0041
Iso+Leu	0	0	0	0	0	0
Phe	0.006	0.007	0.011	0.011	0.018	0.015
Trp	0.018	0.193	0.181	0.150	0.129	0.134
ΣC_{AA}, kg m^{-3}	0.086	0.216	0.217	0.178	0.172	0.172

Table 4.16 The Variation in Extracellular Amino acid Profiles at T=55°C, N=250 min^{-1}, Q_0/V_R=0.5 vvm, pHo=5 controlled

t, h	C_{Asn}	C_{Asp}	C_{Glu}	C_{Gly}	C_{Ser}	C_{His}	C_{Arg}	C_{Thr}	C_{Ala}	C_{Pro}	C_{Tyr}	C_{Val}	C_{Cys}	$C_{Iso+Leu}$	C_{Phe}	C_{Trp}	ΣC_{AA}, kg m^{-3}
0	0.03	0.002	0.003	0.012	0	0	0	0	0	0	0.004	0	0.01	0	0.006	0.02	0.086
3	0.014	0	0	0.009	0	0	0	0	0	0	0	0	0	0.006	0	0.11	0.135
6	0.014	0	0	0	0	0	0	0	0	0	0	0	0	0.005	0.002	0.21	0.235
9	0.01	0	0	0.006	0	0	0	0	0	0	0	0	0	0	0.002	0.27	0.289
12	0.006	0	0	0.004	0	0	0	0	0	0	0	0	0	0	0.003	0.15	0.163
15	0.008	0	0.005	0.003	0	0	0	0	0	0	0	0	0	0.0035	0.002	0.17	0.19
17	0.01	0	0.009	0	0	0	0	0	0	0	0	0	0	0	0.002	0.3	0.325
20	0.01	0	0.009	0	0	0	0	0	0	0	0	0	0	0	0.002	0.3	0.325
24	0.006	0	0.014	0	0	0	0	0	0	0	0	0	0	0	0	0.16	0.177

Table 4.17 The Variation in Extracellular Amino acid Profiles at T=55°C, N=250 min^{-1}, Q_0/V_R=0.2 vvm, pHo=5 controlled

t, h	C_{Asn}	C_{Asp}	C_{Glu}	C_{Gly}	C_{Gln}	C_{Ser}	C_{His} & C_{Arg}	C_{Thr}	C_{Ala}	C_{Pro}	C_{Tyr}	C_{Val}	C_{Cys}	$C_{Iso+Leu}$	C_{Phe}	C_{Trp}	ΣC_{AA}, kg m^{-3}
0	0	0.03	0.002	0.003	0.01	0	0	0	0	0	0.004	0	0.01	0	0.006	0.02	0.086
3	0.01	0	0	0	0	0.011	0	0	0	0	0	0	0	0.006	0.003		0.029
6	0.01	0	0	0	0.04	0	0	0	0	0	0	0	0	0.005	0.002	0.26	0.31
9	0.01	0	0	0	0	0.009	0	0	0	0	0	0	0	0.003	0.001	0.26	0.284
12	0.01	0	0	0	0	0.01	0	0	0	0	0	0	0	0.004	0.002	0.29	0.308
15	0.01	0	0	0	0.03	0	0	0	0	0	0	0	0	0.004	0	0.22	0.258
17	0.01	0	0	0	0	0.012	0	0	0	0	0	0	0	0.005	0.001	0.31	0.345
20	0	0	0	0.01	0	0	0	0	0	0	0	0	0	0.003	0.002	0.23	0.245
24	0.01	0	0	0	0.03	0	0	0	0	0	0	0	0	0.004	0	0.32	0.36

4.4.6 Extracellular Organic Acid Profiles

The variations in extracellular organic acid concentrations with The The variations of organic acid concentrations wrt. cultivation time, air inlet rate, agitation rate and pH strategy are given in Tables 4.18, 4.19, 4.20, 4.21, 4.22, 4.23. When fructose, the glycolysis pathway metabolite was used as the substrate, pH decreases due to the H^+ ion generation in the glycolysis pathway and formation of the organic acids. At all bioreactor conditions acetic acid coming form the precultivation medium was exist in the broths throughout the bioprocesses. The highest and the lowest total organic acid concentration were obtained at LOT-pH_C as 0.46 kg m^{-3} at t=17 h and at LOT-pH_{UC} as 0.135 kg m^{-3} at t=10 h of the bioprocesses, respectively.

At MOT-pH_C succinic acid was excreted in addition to acetate while at LOT-pH_C and LimOT-pH_C lactic acid was excreted due to lack of oxygen. At MOT-pH_C, maximum ΣC_{OA} was 0.43 kg m^{-3} at t=17 h of the bioprocess. At LOT-pH_C , maximum ΣC_{OA} was 0.46 kg m^{-3} at t=17 h of the bioprocess.At LimOT-pH_C, , maximum ΣC_{OA} was 0.34 kg m^{-3} at t=24 h of the bioprocess.

ΣC_{OA} increased with increasing agitation rate-or amount of oxygen in the medium in uncontrolled-pH conditions. At MOT-pH_{UC} and HOT-pH_{UC} in addition to acetic acid, gluconic acid and α-ketoglutarate were excreted respectively. At LOT-pH_{UC} , there was only acetic acid in the fermentation browth. At LOT-pH_{UC}, , the highest ΣC_{OA} was 0.135 kg m^{-3} at t=10 h of the bioprocess. at MOT-pH_{UC} ΣC_{OA} = 0.28 kg m^{-3} at t=20 h of the bioprocess, and at HOT-pH_{UC} , ΣC_{OA}= 0.35 kg m^{-3} at t=45 h of the bioprocess.

Similar to the amino acid profile, at MOT and LOT conditions, pH control produced more organic acids.

Table 4.18 The variations in organic acid concentrations in the fermentation broth with cultivation time, T=55°C, N=500 min^{-1}, Q_0/V_R=0.5 vvm, pHo=5 uncontrolled

C_{OA}, kg m^{-3}	Cultivation time, h				
	t=0	t=10	t=20	t=30	t=40
Cit	0	0	0	0	0
α-Kg	0	0	0	0	0
Suc	0	0	0.198	0	0
Pru	0	0	0	0	0
Ac	0.13	0.13	0	0	0.0045
Lac	0	0.084	0.079	0.015	0
But	0	0	0	0	0
Glu	0	0	0	0	0
Gluc	0	0.0093	0	0	0
ΣC_{OA} , kg m^{-3}	0.13	0.223	0.277	0.015	0.0045

Table 4.19 The variations in organic acid concentrations in the fermentation broth with cultivation time, T=55°C, N=250 min^{-1}, Q_0/V_R=0.5 vvm, pHo=5 uncontrolled

C_{OA}, kg m^{-3}	Cultivation time, h				
	t=0	t=10	t=20	t=29.5	t=45
Cit	0	0	0	0	0
α-kG	0	0	0	0	0
Suc	0	0	0	0	0
Pru	0	0	0	0	0
Ac	0.13	0.135	0.01	0	0
Lac	0	0	0	0	0
But	0	0	0	0	0
Glu	0	0	0	0	0
Gluc	0	0	0	0	0
ΣC_{OA} , kg m^{-3}	0.13	0.135	0.01	0	0

Table 4.20 The variations in organic acid concentrations in the fermentation broth with cultivation time, T=55°C, N=750 min^{-1}, Q_0/V_R=0.5 vvm, pHo=5 uncontrolled

C_{OA}, kg m^{-3}	Cultivation time, h				
	t=0	t=15	t=20	t=27	t=45
Cit	0	0	0	0	0
α-kG	0	0	0	0.062	0
Suc	0	0	0	0	0.27
Pru	0	0	0	0	0
Ac	0.13	0	0.081	0	0.078
Lac	0	0.014	0.0077	0.009	0
But	0	0	0	0	0
Glu	0	0	0	0.136	0
Gluc	0	0	0	0	0
ΣC_{OA} , kg m^{-3}	0.13	0.014	0.088	0.207	0.348

Table 4.21 The variations in organic acid concentrations in the fermentation broth with cultivation time, T=55°C, N=500 min^{-1}, Q_0/V_R=0.5 vvm, pHo=5 controlled

C_{OA}, kg m^{-3}	Cultivation time, h				
	t=0	t=13.5	t=17	t=26	t=40
Cit	0	0	0	0	0
α-kG	0	0	0	0	0
Suc	0	0.137	0.332	0.188	0
Pru	0	0	0	0	0
Ac	0.13	0.048	0.099	0.098	0.065
Lac	0	0	0	0	0
But	0	0	0	0	0
Glu	0	0	0	0.01	0
Gluc	0	0	0	0	0
ΣC_{OA} , kg m^{-3}	0.13	0.185	0.431	0.296	0.065

Table 4.22 The variations in organic acid concentrations in the fermentation broth with cultivation time, T=55°C, N=250 min^{-1}, Q_0/V_R=0.5 vvm, pHo=5 controlled

C_{OA}, kg m^{-3}	Cultivation time, h				
	t=0	t=6	t=9	t=15	t=17
Cit	0	0	0	0	0
α-kG	0	0	0	0	0
Suc	0	0	0	0	0
Pru	0	0	0	0	0
Ac	0.13	0.068	0.182	0	0.457
Lac	0	0.14	0	0	0
But	0	0	0	0	0
Glu	0	0	0	0	0
Gluc	0	0	0	0	0
ΣC_{OA} , kg m^{-3}	0.13	0.208	0.182	0	0.457

Table 4.23 The variations in organic acid concentrations in the fermentation broth with cultivation time, T=55°C, N=250 min^{-1}, Q_0/V_R=0.2 vvm, pHo=5 controlled

C_{OA}, kg m^{-3}	Cultivation time, h				
	t=0	t=6	t=12	t=17	t=24
Cit	0	0	0	0	0
α-kG	0	0	0	0	0
Suc	0	0	0	0	0
Pru	0	0	0	0	0
Ac	0.13	0.116	0.178	0.261	0.336
Lac	0	0.107	0	0	0
But	0	0	0	0	0
Glu	0	0	0	0	0
Gluc	0	0	0	0	0
ΣC_{OA} , kg m^{-3}	0.13	0.223	0.178	0.261	0.336

4.4.7 Specific Growth Rate and Maintenance Coefficients

In this study, since Trp was the aromatic amino acid produced at the highest amount and frequency, it was considered as the main product. Thus yield coefficients based on Trp were calculated in order to understand the production metabolism of tryptophan.

The instantaneous and overall yield coefficients for controlled and uncontrolled-pH operations with respect to fructose and oxygen as substrates and the variations in specific growth rate (μ), specific oxygen uptake rate (q_o), specific fructose uptake rate (q_S), specific product formation rate (q_P), are given in Tables 4.24 to 4.35 respectively.

The variations in specific growth rate (μ) with cultivation time, air inlet rate, agitation rate and pH strategy are given in Figures 4.15. All of the curves were logarithmic. Among all bioreactor conditions, the highest and the lowest μ profiles were at MOT-pH_C and LOT-pH_C , respectively. However the profile of LOT-pH_C was slightly lower than LimOT-pH_C. Thus with the increase in oxygen in the medium, μ can be said to increase. Among uncontrolled-pH operations, for the first 15 h of the bioprocesses the lowest μ profile was at LOT-pH_{UC} while the highest μ profiles were at MOT-pH_{UC} and HOT-pH_{UC}. Thus with the increase in agitation rate, μ increased for the first 15 h of the bioprocesses. After those periods, μ got zero values. At MOT condtion μ profiles of uncontrolled and controlled-pH operations were similar for t=5-17 h of the bioprocesses. At LOT condition, μ profiles of uncontrolled and controlled-pH operations were similar.

The variations in specific fructose consumption rate (q_S) with cultivation time, air inlet rate, agitation rate and pH strategy are given in Figures 4.16. Among all bioreactor conditions, the highest q_S values belonged to MOT-pH_C, where the highest cell concentration and fructose consumption were seen. At MOT condtion, controlled-pH operation had higher q_S profile than uncontrolled-pH operation because of the higher metabolic activity at controlled-pH operation. Except t=9 and t=12 h of the bioprocess, LOT-pH_{UC} had higher q_S values than LOT-pH_C . Because at LOT-pH_{UC} , there was higher cell growth although at LOT-pH_C there was higher Trp production . The relation of q_S and cell

concentrations were more connected to the relation of q_S to Trp production. Thus it seems that fructose consumption was not so much related to Trp production, but it directly affected cell growth.

The variations in specific Trp production rate (q_P) with cultivation time, air inlet rate, agitation rate and pH strategy are given in Figures 4.17. Among all bioreactor conditions the highest and the lowest q_P profiles were at LimOT-pH_C and MOT-pH_{UC} operations, respectively; indicating that specific productivity of Trp favored the lowest-oxygen transfer conditions. Also lowest Trp production was obtained at MOT-pH_{UC} condition though the cell concentration was high. Thus it had the lowest q_P profile. At controlled pH operations the highest and the lowest q_P profiles were at LimOT-pH_C and MOT-pH_C, indicating that with the increase in oxygen in the medium, the specific production rate of Trp decreased. Among uncontrolled-pH operations, the lowest q_P profile was at MOT-pH_{UC}. LOT-pH_C operation had higher q_P profile than LOT- pH_{UC} operation; because of the fact that Trp production was better at controlled pH operation than uncontrolled one. (MOT lar karsilastirilamiyor).

The variations in specific oxygen consumption rate (q_O) with cultivation time, air inlet rate, agitation rate and pH strategy are given in Figures 4.18. Among all bioreactor operations, the highest and the lowest q_O profiles were at LimOT-pH_C and HOT-pH_{UC} operations respectively.This result shows that as the oxygen in the medium decreased, q_O decreased. At controlled-pH operations, the highest and the lowest q_O profiles were at LimOT-pH_C and MOT-pH_C operations respectively. Thus with the increase in oxygen in the medium, q_O decreased. At uncontrolled pH operations, the highest and the lowest q_O profiles were uncomparable. At LOT condition, controlled pH profile had slightly higher values than uncontrolled one. At MOT condition, for the t=3- 15 h of the bioprocess, uncontrolled-pH condition had higher q_O values than controlled-pH operations.

At controlled-pH operations, the lowest $Y_{X/S}$ values were obtained at MOT-pH_C operation throughout the fermentation inspite of the simultaneously high cell concentration and fructose consumption profiles. At LOT-pH_C operation, for the first 6 h of the bioprocess, $Y_{X/S}$ values were higher than that of LimOT-pH_C

operation. At uncontrolled-pH operations, the higher $Y_{X/S}$ values were obtained at LOT-pH_{UC} compared to the MOT-pH_{UC} and HOT-pH_{UC} because of the shift of the metabolism towards to the by-product (organic acid) formation at higher agitation rates.

At controlled-pH operations, $Y_{P/S}$ values were the highest at LimOT-pH_C, as the lowest fructose consumption and the highest Trp production was obtained at LimOT-pH_C showing the carbon flow towards aromatic pathway under oxygen limited conditions. At controlled-pH operations, $Y_{P/S}$ values were the lowest at MOT-pH_C ; as Trp production was lowest and fructose consumption was highest at that condition among all controlled-pH operation, indicating that carbon flux flowed tp cell generation rather than Trp generation. At uncontrolled-pH operations, the highest and the lowest $Y_{P/S}$ profiles were at LOT-pH_{UC} and MOT-pH_{UC} , respectively. At both LOT and MOT conditions, controlled-pH cases gave higher $Y_{P/S}$ values than uncontrolled ones; showing there was more carbon flow towards aromatic pathway under controlled-pH operations.

At controlled-pH operations, for t=0-6 h, LimOT-pH_C operation had higher $Y_{P/X}$ profile than LOT-pH_C operation. However after t=6 h of the bioprocess, LOT-pH_C condition had higher $Y_{P/X}$ profile than LimOT-pH_C condition. Among controlled-pH operations, MOT-pH_C had the lowest $Y_{P/X}$ profile because of the existences of both the highest cell concentration and the lowest Trp production at MOT-pH_C . Among uncontrolled operations, the highest and the lowest $Y_{P/X}$ profiles were at LOT-pH_{UC} and MOT-pH_{UC} , respectively. At both LOT and MOT conditions, controlled-pH cases gave higher $Y_{P/X}$ values than uncontrolled ones indicating the shift of carbon flow from by-product formation to product formation.

Among controlled-pH operations, the highest $Y_{X/O}$ values were obtained at MOT-pH_C indicating that the oxygen is efficiently utilized in the metabolism. For the first t=0-12 h of the bioprocess, $Y_{X/O}$ profile was higher at LOT-pH_C than LimOT-pH_C. Namely among controlled-pH operations lowest $Y_{X/O}$ values were observed at the first 12 h of LimOT-pH_C due to the lowest cell growth obtained at this operation. Afterwards, $Y_{X/O}$ profile was higher at LimOT-pH_C than LOT-pH_C. At LOT condition, controlling pH gave lower $Y_{X/O}$ values than

uncontrolled-pH conditions as there was lower cell growth at uncontrolled operation of LOT. However at MOT condition, controlling pH gave higher $Y_{X/O}$ values, as there was higher cell growth at controlled-pH operation of MOT.

Among controlled-pH operations, highest $Y_{S/O}$ values were at MOT-pH_C, due to the highest fructose consumption rate; although it had the highest oxygen uptake rate among all controlled operations. For the first t=0-6 h of the bioprocess at LOT, controlled-pH condition had higher $Y_{S/O}$ values than uncontrolled-pH condition indicating the utilization of the carbon source in the non-oxidative pathways and also energy generation by the substrate level phosphorylation. A high $Y_{S/O}$, indicating a preference of utilization of substrate over oxygen can be good in terms of Trp production as the carbon flux does not so much move to TCA cycle but it moves to non-oxidative pathways that are related with Trp production; however it might not be good for producing a higher amount of ATP. A low value of $Y_{S/O}$ indicates more energy production by TCA cycle rather than substrate level phosphorylation thus it indicates higher energy generation though it may not be good for Trp production.

Among controlled-pH operations, lowest $Y_{P/O}$ values were obtained at MOT-pH_C, while the highest $Y_{P/O}$ values were at LimOT-pH_C showing that Trp pathway fluxes was favored by the oxygen limited conditions. Among uncontrolled-pH operations, lowest $Y_{P/O}$ values were at HOT-pH_{UC}, while highest $Y_{P/O}$ values were at LOT-pH_{UC}. Thus $Y_{P/O}$ values decreased with increasing agitation rates at uncontrolled-pH operations. This was reasonable as at higher agitation rates, carbon flux moved to oxidative pathways rather than Trp production pathway. At LOT, controlled and uncontrolled-pH conditions had similar $Y_{P/O}$ values. At MOT, controlled-pH condition had higher $Y_{P/O}$ values than uncontrolled-pH condition.

Maintenence coefficient is the energy expenditure for maintenance processes per unit cell formed. In engineering point of view, maintenence coefficient is wanted to be minimized to maximize cell growth and product formation to increase the product and cell yields on substrate. Thus maintenance coefficients were calculated in order to have an idea on the amount of carbon fluxes moving maintenance processes rather than product, by-product and

cell formations. Thus it gives an idea on the efficiency of the bacteria for energy utilization. In order to find maintenance coefficients, firstly the product formation was related with the cell formation according to the model of Leudeking Piret. Leudeking-Piret constants, α and β, found for the bioreactor experiments are given in Tables 4.38 and 4.39, respectively. α is the term for the synthesis of growth associated products while, β is the term for production of non-growth associated products that are synthesized in stationary phase of the growth. In this context, the reason why β had negative values at LimOT-pH_C, LOT-pH_C, MOT-pH_C and LOT-pH_{UC} is that at each condition, the Trp production had already reached its maximum concentration, before the cell concentration reached stationary phase. After the maximum concentration of Trp is reached, Trp was started to be utilized by the bacteria-during the rest of its exponential growth period and the stationary growth period. A better model relating Trp production to cell growth should be made in the future.

The maintenance coefficients for oxygen (mo) and fructose (ms) as the substrates were given in Tables 4.36 and 4.37. During the calculation of mo in the periods where C_{DO} was zero, it was assumed that after the oxygen depleted, k_La values were equal to the average k_La values obtained in the period starting with t=0 and finishing with the time when C_{DO} became zero. The periods when this assumption were used , were denoted by * in Tables 4.24 to 4.35, respectively. Thus $Y_{X/O}$, $Y_{P/O}$, $Y_{S/O}$ values for those periods and overall maintenance coefficients on oxygen could be determined. This was applied to LOT-pH_{UC} starting from t=5, to LOT-pH_C starting from t=5 and finally to LimOT-pH_C starting from t=3.

At controlled-pH operations, with the decrease in Q_O/V_R and/or N maintenance coefficients for substrate increased probably because of the ATP, used for the transportation, translocation and maintenance of the gradient, was generated by the substrate level phosphorylation. Similarly, at controlled-pH operations, with the decrease in Q_O/V_R and/or N maintenance coefficients for oxygen increased as the available oxygen was used for the energy generation by the oxidative phosphorylation which was coupled with the proton electrochemical gradient.

At uncontrolled-pH operations, highest and lowest mo were at MOT-pH_{UC} and LOT-pH_{UC} operations.

At uncontrolled-pH operations, highest and lowest ms were at MOT-pH_{UC} and LOT-pH_{UC} operations. At uncontrolled-pH operations, lower oxygen transfer conditions produced both higher Trp and higher cell concentration; thus lowering ms by lowering the carbon flux through maintenance processes.

At LOT- pH_C ms was higher than LOT-pH_{UC} . This might be due to the lower cell formation at LOT-pH_C indicating that carbon flux moved to maintenance processes rather than cell formation. At MOT- pH_C ms was lower than MOT-pH_{UC} . This might be due to the higher cell formation at MOT-pH_C indicating that carbon flux moved to cell formation rather than maintenance processes.

Table 4.24 The variations in specific growth rate, μ and yield coefficients at LimOT-pH_C with fructose as substrate

t	μ	q_S	q_P	$Y_{X/S}$	$Y_{S/O}$	$Y_{P/S}$	$Y_{P/X}$
(h)	(h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})
0.13	0.82	2.79	1.18	0.29	2.7	0.42	1.4
0.0817	0.53	1.78	0.75	0.29	2.0	0.42	1.4
3*	0.23	0.83	0.35	0.28	0.85	0.42	1.5
6*	0.10	0.50	0.11	0.20	0.85	0.22	1.1
9*	0.06	0.24	0.02	0.23	0.50	0.08	0.37
12*	0.04	0.07	-	0.68	0.16	-	-
15*	0.03	0.06	0.04	0.49	0.16	0.71	1.4
17*	0.02	0.24	0.03	0.10	0.69	0.13	1.2
20*	0.02	0.39	-	0.06	1.2	-	-
Overall				0.18		0.15	0.87

Table 4.25 The variations in specific growth rate, μ and yield coefficients at LOT-pH_C with fructose as substrate

t	μ	q_S	q_P	$Y_{X/S}$	$Y_{S/O}$	$Y_{P/S}$	$Y_{P/X}$
(h)	(h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})
0.8	0.68	1.1	0.45	0.61	1.6	0.40	0.66
1.8	0.41	0.67	0.27	0.61	0.90	0.40	0.66
2.6	0.30	0.49	0.20	0.61	0.90	0.40	0.66
3	0.32	0.44	0.20	0.73		0.45	0.62
6*	0.07	0.21	0.08	0.33	0.37	0.38	1.1
9*	0.08	0.90	-	0.08	2.3	-	-
12*	0.04	0.80	-	0.05	2.4	-	-
15*	0.01	0.19	0.06	0.06	0.63	0.30	4.9
17*	0.005	0.21	0.05	0.02	0.67	0.24	10.8
20*	0.005	0.22	-	0.03	0.72	-	-
Overall				0.13		0.03	0.22

Table 4.26 The variations in specific growth rate, μ and yield coefficients at MOT-pH_C with fructose as substrate

t	μ	q_S	q_P	$Y_{X/S}$	$Y_{S/O}$	$Y_{P/S}$	$Y_{P/X}$
(h)	(h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})
0.5	0.74	8.53	0.28	0.086	16.7	0.034	0.39
2	0.62	3.98	0.13	0.15		0.034	0.22
3	0.54	2.4	0.08	0.22	3.3	0.034	0.15
5	0.32	1.34	0.04	0.23		0.027	0.11
13.5	0.06	0.26	0.003	0.24	3.13	0.012	0.05
14.5	0.02	0.16	-	0.1		-	-
17.3	0	-	-	-	-	-	-
Overall				0.228		0.02	0.1

Table 4.27 The variations in specific growth rate, μ and yield coefficients at LimOT-pH_C with oxygen as substrate

t	μ	q_O	q_P	$Y_{X/O}$	$Y_{S/O}$	$Y_{P/O}$	$Y_{P/X}$
(h)	(h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})
0.13	0.82	1.0	1.2	0.80	2.7	1.1	1.4
0.82	0.53	0.87	0.75	0.60	2.0	0.86	1.4
3*	0.23	0.97	0.35	0.24	0.85	0.36	1.5
6*	0.10	0.59	0.11	0.17	0.85	0.19	1.1
9*	0.06	0.48	0.02	0.12	0.50	0.04	0.36
12*	0.04	0.42	-	0.11	0.16	-	-
15*	0.03	0.36	0.04	0.08	0.16	0.11	1.5
17*	0.02	0.35	0.03	0.07	0.69	0.09	1.2
20*	0.02	0.32	-	0.08	1.2	-	-
Overall							0.87

Table 4.28 The variations in specific growth rate, μ and yield coefficients at LOT-pH_C with oxygen as substrate

t	μ	q_O	q_P	$Y_{X/O}$	$Y_{S/O}$	$Y_{P/O}$	$Y_{P/X}$
(h)	(h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})
0.8	0.68	0.70	0.45	0.97	1.6	0.63	0.66
1.8	0.41	0.75	0.27	0.55	0.9	0.36	0.66
2.6	0.30	0.55	0.20	0.55	0.9	0.36	0.66
3	0.32	-	0.20				0.62
6*	0.07	0.56	0.08	0.12	0.37	0.14	1.1
9*	0.08	0.39	-	0.20	2.3	-	-
12*	0.04	0.33	-	0.11	2.4	-	-
15*	0.01	0.31	0.06	0.04	0.63	0.19	5.0
17*	0.005	0.31	0.05	0.01	0.67	0.16	10.8
20*	0.006	0.30	-	0.02	0.72	-	-
Overall							0.22

Table 4.29 The variations in specific growth rate, μ and yield coefficients at MOT-pH_C with oxygen as substrate

t	μ	q_O	q_P	$Y_{X/O}$	$Y_{S/O}$	$Y_{P/O}$	$Y_{P/X}$
(h)	(h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})
0.5	0.74	0.51	0.28	1.4	16.7	0.56	0.39
2	0.62		0.13				0.22
3	0.54	0.72	0.08	0.75	3.3	0.11	0.15
5	0.32		0.4				0.11
13.5	0.06	0.08	0.003	0.76	3.1	0.04	0.05
14.5	0.02		-				-
17.3	0						-
Overall							0.1

Table 4.30 The variations in specific growth rate, μ and yield coefficients at LOT-pH_{UC} with fructose as substrate

t	μ	q_S	q_P	$Y_{X/S}$	$Y_{S/O}$	$Y_{P/S}$	$Y_{P/X}$
(h)	(h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})
0.67	0.76	2.1	0.34	0.36	3.7	0.16	0.45
1.5	0.45	1.3	0.21	0.36	2.2	0.16	0.45
2	0.38	1.1	0.17	0.36	1.8	0.16	0.45
3.5	0.24	0.67	0.11	0.35		0.16	0.46
5*	0.19	0.51	0.08	0.38	0.87	0.16	0.42
6*	0.15	0.41	0.07	0.35	0.87	0.16	0.46
8*	0.08	0.42	0.05	0.20	1.1	0.13	0.65
10*	0.05	0.42	0.01	0.13	1.2	0.03	0.23
20*	0.02	0.25		0.09	1		
22*	0.02	0.47		0.05	2.0		
24.5*	0.003	0		-			
27*	0.005	0.004		-	0.02		
29.5*	0.02	0.22		0.02	0.99		
40*	0.02	0.11		0.16	0.59		
Overall				0.12		0.01	0.08

Table 4.31 The variations in specific growth rate, μ and yield coefficients at MOT-pH_{UC} with fructose as substrate

t	μ	q_S	q_P	$Y_{X/S}$	$Y_{S/O}$	$Y_{P/S}$	$Y_{P/X}$
(h)	(h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})
2	0.30	2.68	0.08	0.11	-	0.03	0.27
5	0.25	2.1	0.04	0.12	2.9	0.02	0.18
6	0.25	1.5	0.03	0.16		0.02	0.13
7	0.20	1.2	0.03	0.16		0.02	0.13
8	0.25	0.95	0.02	0.26	1.7	0.02	0.09
9	0.25	0.66	0.02	0.38		0.02	0.06
10	0.17	0.52	0.007	0.32		0.01	0.04
11	0.12	0.46	0.001	0.25		0.003	0.01
12	0.09	0.41	0.001	0.23		0.003	0.01
13	0.08	0.38	0.001	0.2		0.003	0.01
14	0.03	0.35	0.0009	0.07	1.3	0.003	0.04
15	0.03	0.36	0.0009	0.09		0.003	0.03
17	0.04	0.31	0.0008	0.12		0.003	0.02
20	0	0.21	0.002	0	0.95	0.01	
25	0	0.04	0.007	0			
30	0	0.04	0.002	0		0.06	
35	0	0.04	0.0002	0		0.005	
40	0	0.01	0.001	0		0.09	
Overall				0.14		0.019	0.14

Table 4.32 The variations in specific growth rate, μ and yield coefficients at HOT-pH_{UC} with fructose as substrate

t	μ	q_S	q_P	$Y_{X/S}$	$Y_{S/O}$	$Y_{P/S}$	$Y_{P/X}$
(h)	(h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})
2	0.32	1.4	0.15	0.23	2.1	0.1	0.46
3	0.31	1.1	0.11	0.28	1.3	0.1	0.36
15	0	0.32	-	0.10		-	
16	0	0.44	-	0	2.5	-	
17	0	0.44	-	0		-	
20	0	0.32	-	0		-	
25	0	0.55	0.02	0		0	
27	0	0.48	0.01	0		0	
30	0	0.08	0.002	0		0.03	
40	0		0.002			0.02	
42	0		0.002			0.03	
Overall				0.1		0.04	0.36

Table 4.33 The variations in specific growth rate, μ and yield coefficients at LOT-pH_{UC} with oxygen as substrate

t	μ	q_O	q_P	$Y_{X/O}$	$Y_{S/O}$	$Y_{P/O}$	$Y_{P/X}$
(h)	(h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})
0.67	0.76	0.58	0.34	1.3	3.7	0.59	0.45
1.5	0.45	0.58	0.21	0.78	2.2	0.36	0.45
2	0.38	0.58	0.17	0.66	1.8	0.30	0.45
3.5	0.24		0.11				0.46
5*	0.20	0.59	0.08	0.33	0.87	0.14	0.42
6*	0.15	0.48	0.07	0.31	0.87	0.14	0.45
8*	0.08	0.39	0.05	0.21	1	0.14	0.65
10*	0.05	0.34	0.01	0.16	1.2	0.04	0.23
20*	0.02	0.24		0.09	1		
22*	0.02	0.24		0.1	2		
24.5*	0.02	0.22		0.02			
27*	0.003	0.22		0.02	0.02		
29.5*	0.005	0.22		0.02	0.99		
40*	0.02	0.18		0.1	0.59		
Overall							0.08

Table 4.34 The variations in specific growth rate, μ and yield coefficients at MOT-pH_{UC} with oxygen as substrate

t	μ	q_O	q_P	$Y_{X/O}$	$Y_{S/O}$	$Y_{P/O}$	$Y_{P/X}$
(h)	(h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})
2	0.30	-	0.08	-	-	-	0.27
5	0.25	0.71	0.04	0.35	2.9	0.06	0.17
6	0.25		0.03				0.13
7	0.20		0.03				0.13
8	0.25	0.55	0.02	0.46	1.7	0.04	0.09
9	0.25		0.02				0.06
10	0.17		0.007				0.04
11	0.12		0.001				0.01
12	0.09		0.001				0.01
13	0.08		0.001				0.01
14	0.03	0.27	0.0009	0.1	1.3	0.003	0.03
15	0.03		0.0009				0.03
17	0.04		0.0008				0.02
20	0	0.22	0.002	0	0.95	0.01	
25	0		0.007				
30	0		0.002				
35	0		0.0002				
40	0		0.0014				
Overall							0.14

Table 4.35 The variations in specific growth rate, μ and yield coefficients at HOT-pH_{UC} with oxygen as substrate

t	μ	q_O	q_P	$Y_{X/O}$	$Y_{S/O}$	$Y_{P/O}$	$Y_{P/X}$
(h)	(h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1} h^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})	(kg kg^{-1})
2	0.32	0.68	0.15	0.47	2.1	0.22	0.46
3	0.31	0.84	0.11	0.36	1.3	0.13	0.36
15	0.03		-		0		
16	0	0.17	-	0	2.5		
17	0		-				
Overall							0.36

Table 4.36 Variation of maintenance coefficients based on oxygen (mo) and fructose (ms) as the substrates at controlled-pH conditions

Bioreactor Operation Condition	mo (kg kg^{-1} h^{-1})	ms (kg kg^{-1} h^{-1}) and its period
LimOT-pH_C	0.33, t=0.133-20 h	0.364, t=0.13-20 h
LOT-pH_C	0.34, t=0.8-20 h	0.295, t=0.8-20 h
MOT-pH_C	1.28, t=0.5-3 h	0.12, t=3-14.5 h

Table 4.37 Variation in maintenance coefficients based on oxygen (mo) and fructose (ms) at uncontrolled-pH conditions

Bioreactor Operation Condition	mo (kg kg^{-1} h^{-1})	ms (kg kg^{-1} h^{-1}) and its period
LOT-pH_{UC}	0.233, t=0.67-40 h	0.255, t=0.67-29.5 h
MOT- pH_{UC}	0.198, t=5-14 h	0.284, t=9-17 h
HOT- pH_{UC}	-	0.206, t=2-15 h

Table 4.38 Leudeking-Piret constants in controlled-pH condtions

Bioreactor Operation Condition	α (g Trp / g cell)	B (g Trp / (g cell.hr))
LimOT-pH_C	1.52	-0.038
LOT-pH_C	0.92	-0.038
MOT-pH_C	0.115	-0.0011

Table 4.39 Leudeking-Piret constants in uncontrolled-pH condtions

Bioreactor Operation Condition	α (g Trp / g cell)	B (g Trp / (g cell.hr))
LOT-pH_{UC}	0.62	-0.0174
MOT-pH_{UC}	0.067	0.002
HOT-pH_{UC}	0.314	0.00124

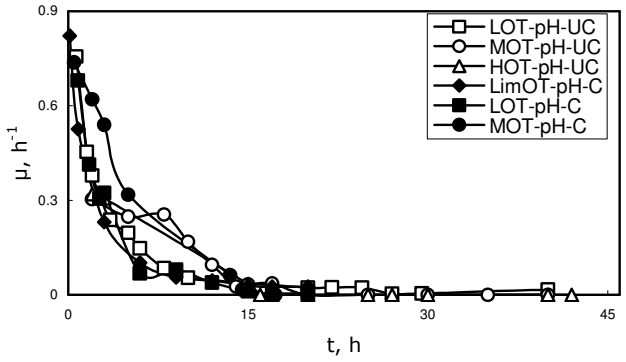


Figure 4.15 The variations in specific growth rate profiles with the cultivation time, air inlet rate, agitation rate and pH strategy

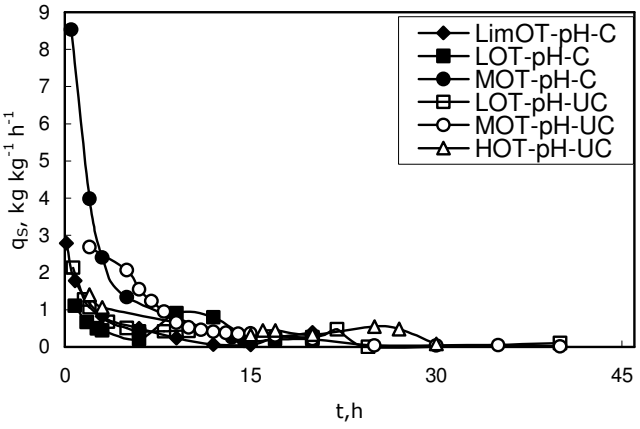


Figure 4.16 The variations in specific fructose consumption rate profiles with the cultivation time, air inlet rate, agitation rate and pH strategy

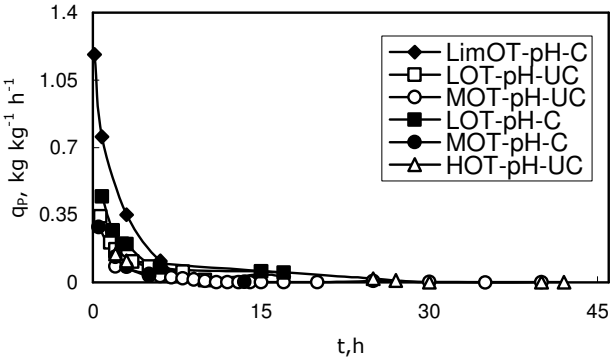


Figure 4.17 The variations in specific Trp production rate profiles with the cultivation time, air inlet rate, agitation rate and pH strategy

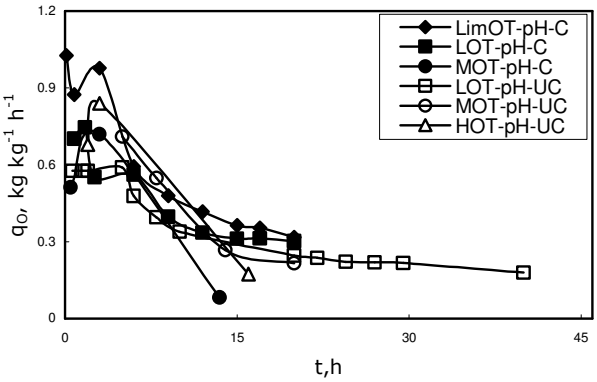


Figure 4.18 The variations in specific oxygen consumption rate profiles with the cultivation time, air inlet rate, agitation rate and pH strategy

4.4.8 Oxygen Transfer Characteristics

The dynamic method was applied to find the oxygen transfer parameters, i.e., oxygen uptake rate (OUR), r_0, and oxygen transfer coefficient, K_La, during the cultivation times corresponding to the characteristic regions of the batch bioprocess. At t=0 h, the oxygen transfer coefficient K_La_o values were measured in the medium in the absence of the microorganism. When C_{DO} decreased to values below % 20 of the saturation values, the dynamic method are not applied not to affect the metabolism of the microorganism. The variations in K_La, oxygen uptake rate, oxygen transfer rate and the enhancement factor E ($=K_La/K_La_o$) throughout the growth phase of the bioprocess are given in Table 4.40 to Table 4.45.

4.4.8.1 Oxygen Transfer Parameters

For $LimOT_C$, LOT_C and LOT_{UC} , C_{DO} values were diminished at t=3, 5 and 5 h of the bioprocesses respectively. Thus in the calculation of OUR and maintenances, as it has been said previously, it was assumed that after the oxygen depleted, k_La values were equal to the average k_La values obtained in the period starting with t=0 and finishing with the time when C_{DO} became zero. In this context, in the periods when $C_{DO}=0$, OTR=OUR and the time in which the above assumption was applied were denoted with * in tables 4.40 and 4.45.

At uncontrolled-pH operations, MOT and HOT conditions, have parabolic curves of oxygen uptake rate (OUR) vs. time (Figure 4.15) as oxygen consumption first increase with the increase in metabolic activities in the exponential phase and then decrease because of the retardation of the metabolic activities.

Due to the depletion of oxygen at LOT-pH_{UC} at t= 5 h, dynamic method could not be applied after this hour; however, trend of the uncontrolled-pH operations OUR profiles showed that OUR was inversely proportional to the agitation rate indicating the inhibitory effect of high dissolved oxygen concentrations on the metabolism. Higher agitation rates might cause less

oxygen uptake and thus carbon fluxes might flow more into non-oxidative pathways.

At MOT condition, controlled-pH operation had higher oxygen uptake rates than the uncontrolled one at beginning of the bioprocess due to the higher metabolic activities. Similarly, at LOT, controlled case had higher oxygen uptake rates.

In controlled-pH operations, at MOT-pH_C K_La firstly increased and then decreased during for t=0-13.5 h of the bioprocess , where K_La values changed in the interval of 0.016-0.046 (Figure 4.16). At LOT-pH_C , K_La increased during t=0-2.6 h of the bioprocess. K_La values were in the range of 0.0083-0.012 . At LimOT-pH_C , K_La increased during t=0-1 h of the bioprocess, with the range of 0.0069-0.004. For $LimOT_C$ and LOT_C conditions, C_{DO} values were diminished after t=2.5 and t= 4 h of the bioprocesses respectively. The highest K_La was at MOT-pH_{UC} and the lowest K_La was at $LimOT_C$, as expected.

In uncontrolled-pH operations, maximum K_La increased with increasing agitation rate. The K_La profiles of uncontrolled-pH conditions were variable. The range of K_La were 0.0059-0.0081 at LOT-pH_{UC} , 0.0183-0.02978 at MOT-pH_{UC} and 0.04-0.045 at HOT-pH_{UC}.

At both MOT and LOT conditions, controlling pH gave higher maximum K_La values than uncontrolled-pH condition. When gas-liquid mass transfer accompanied by a chemical reaction, mass transfer can be increased several fold as a result of the relative rates of mass transfer and chemical reaction and as the metabolic activities were higher under controlled conditions, higher K_La values were obtained. Also at MOT-pH_C and LOT-pH_C conditions, maximum ΣC_{AA} , ΣC_{OA} in the media and OH^- concentrations of the media were higher in the medium. More amino, organic acids and more OH^- ions in the media might mean a more polar environment in the fermentation broth, changing reological properties of the medium, changing the attractive forces between the molecules. The increased polarity or ion dipoles in the medium might have attracted and caused higher transport of oxygen-which is also polar- from gas bubbles into the media the increase in the K_La. In order to

find the rate limiting step and the degree of the limitation in the bioprocess of *B. acidocaldarius*, the maximum possible oxygen utilization rate ($OD=\mu_{max}C_X/Y_{X/O}$) and the maximum possible mass transfer rate ($OTR_{max}=K_La C_{DO}^*$) were also calculated throughout the growth phase of the bioprocess. A kind of Damköhler number, Da, defined as maximum possible oxygen utilization rate per maximum mass transfer rate (Çalık et al., 2000 and 2003); and effectiveness factor, η, defined as the oxygen uptake rate per maximum possible oxygen utilization rate values (Çalık et al., 2000 and 2003) can be seen from Tables 4.40 to 4.45.

In all controlled-pH operations, Damköhler number was increasing wrt. time. Thus as the fermentation continued, mass transfer resistances were becoming more effective than biochemical reaction resistances. This is meaningful as the oxygen in the medium falled to very low values at later stages of the fermentation. At LimOT-pH_C condition, for the first 3 hours of the bioprocess, biochemical reaction resistances were more effective than mass transfer resistances. However, after t=3 h, mass transfer resistances became more effective . At LOT-pH_C , for the first 3 hours biochemical reaction resistances were more effective, however after t=6h , mass transfer resistances became more effective . At LimOT-pH_C and LOT-pH_C , for the periods of t>3h, the existance of diffusion limitations were in harmony with the oxygen profiles showing C_{DO} values as zero for those periods.At MOT-pH_C , for the first 3 h, biochemical reaction resistances were more effective. However after 8 h, mass transfer resistances became more effective.

In uncontrolled-pH operations, for the first 2 hours of LOT-pH_{UC}, for the first 8 hours of MOT-pH_{UC} and for the first 16 hours of HOT-pH_{UC} , Damköhler numbers were always smaller than 1, showing that biochemical reaction resistances were more effective. However for MOT-pH_{UC} t=14 h mass-transfer resistances were more effective and for HOT-pH_{UC} t=42 h of the bioprocess, both of the resistances were equally effective.

In all controlled-pH operations, effectiveness factors were 1 at very early hours of the bioprocesses, however by time passed they decreased to lower values. The same thing also occurred for uncontrolled-pH cases.

Table 4.40 The variations in the oxygen transfer parameters at LimOT-pH_C

t (h)	k_La (s^{-1})	E k_La/k_La_0	OTR^x10^3 ($molm^{-3}s^{-1}$)	$OTR_{max}{}^x10^3$ ($molm^{-3}s^{-1}$)	OUR^x10^3 ($molm^{-3}s^{-1}$)	$OD\times10^3$ ($molm^{-3}s^{-1}$)	Da OD/OTRmax	η OUR/OD
0.13	0.01	2.04	0.43	1.22	0.3	0.3	0.25	1
0.82	0	1.17	0.39	0.7	0.4	0.62	0.89	0.65
3*	0.01	1.61	0.96	0.96	0.96	3.41	3.56	0.28
12*	0.01	1.61	0.96	0.96	0.96	17.2	17.9	0.06
20*	0.005	1.61	0.96	0.96	0.96	31	32.4	0.031

Table 4.41 The variations in the oxygen transfer parameters at LOT-pH_C

t (h)	k_La (s^{-1})	E k_La/k_La_0	OTR^x10^3 ($molm^{-3}s^{-1}$)	$OTR_{max}{}^x10^3$ ($molm^{-3}s^{-1}$)	OUR^x10^3 ($molm^{-3}s^{-1}$)	$OD\times10^3$ ($molm^{-3}s^{-1}$)	Da OD/OTRmax	η OUR/OD
0.8	0.01	2.02	0.39	1.45	0.4	0.4	0.28	1
1.77	0.009	2.2	0.67	1.58	0.7	1.15	0.73	0.608
2.6	0.01	2.98	1.34	2.14	0.7	1.55	0.72	0.45
6*	0.01	2.4	1.72	1.72	1.72	16.9	9.82	0.1
12*	0.01	2.4	1.72	1.72	1.72	30.2	17.5	0.06
20*	0.01	2.4	1.72	1.72	1.72	203	118	0.01

Table 4.42 The variations in the oxygen transfer parameters at MOT-pH_C

t (h)	k_La (s^{-1})	E k_La/k_La_0	OTR^x10^3 ($molm^{-3}s^{-1}$)	$OTR_{max}{}^x10^3$ ($molm^{-3}s^{-1}$)	OUR^x10^3 ($molm^{-3}s^{-1}$)	$OD\times10^3$ ($molm^{-3}s^{-1}$)	Da OD/OTRmax	η OUR/OD
0.5	0.02	2.47	0.11	2.98	0.2	0.2	0.07	1
3	0.05	6.68	0.88	8.07	1	1.36	0.17	0.73
8*	0.03	4.49	2	5.42	2	6.57	1.21	0.3
13.5	0.02	2.37	1.23	2.87	1.3	13	4.53	0.1

Table 4.43 The variations in the oxygen transfer parameters at LOT-pH_{UC}

t (h)	k_La (s^{-1})	E k_La/k_La_0	OTR^x10^3 ($molm^{-3}s^{-1}$)	$OTR_{max}{}^x10^3$ ($molm^{-3}s^{-1}$)	OUR^x10^3 ($molm^{-3}s^{-1}$)	$OD\times10^3$ ($molm^{-3}s^{-1}$)	Da OD/OTRmax	η OUR/OD
0.67	0.0081	1.98	0.37	1.42	0.3	0.3	0.21	1
1.55	0.01	1.44	0.38	1.03	0.5	0.83	0.81	0.6
2	0.01	1.9	0.61	1.37	0.6	1.2	0.87	0.5
6*	0.01	1.78	1.27	1.27	1.27	6.56	5.16	0.19
12*	0.01	1.78	1.27	1.27	1.27	20.8	16.4	0.06
20*	0.01	1.78	1.27	1.27	1.27	42.1	33.1	0.03
40*	0.01	1.78	1.27	1.27	1.27	56	44.1	0.02

Table 4.44 The variations in the oxygen transfer parameters at MOT-pH_{UC}

t (h)	k_La (s^{-1})	E k_La/k_La_0	OTR^x10^3 ($molm^{-3}s^{-1}$)	$OTR_{max}{}^x10^3$ ($molm^{-3}s^{-1}$)	OUR^x10^3 ($molm^{-3}s^{-1}$)	$OD\times10^3$ ($molm^{-3}s^{-1}$)	Da OD/OTRmax	η OUR/OD
5	0.03	4.32	0.71	5.21	0.9	0.92	0.18	0.98
8	0.02	3	0.89	3.62	1.4	1.4	0.39	1
14	0.02	3.45	1.27	4.17	1.7	16.3	3.9	0.1
20	0.02	2.65	0.92	3.2	1.5	49.8	15.5	0.03

Table 4.45 The variations in the oxygen transfer parameters at HOT-pH_{UC}

t (h)	k_La (s^{-1})	E k_La/k_La_0	OTR^x10^3 ($molm^{-3}s^{-1}$)	$OTR_{max}{}^x10^3$ ($molm^{-3}s^{-1}$)	OUR^x10^3 ($molm^{-3}s^{-1}$)	$OD\times10^3$ ($molm^{-3}s^{-1}$)	Da OD/OTRmax	η OUR/OD
2	0.045	1.99	0.338	7.87	0.489	0.489	0.062	1
3	0.04	1.77	0.38	7	0.8	0.83	0.12	0.96
16	0.05	2	0.83	7.92	0.9	5.3	0.67	0.17
42	0.04	1.85	1.52	7.31	0.02	14.2	1.95	0

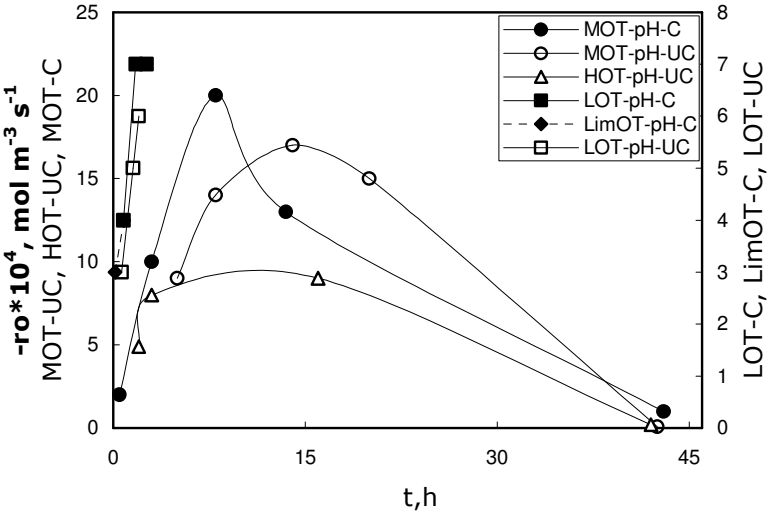


Figure 4.19 The variations in oxygen uptake rate profiles with the cultivation time, air inlet rate, agitation rate and pH strategy

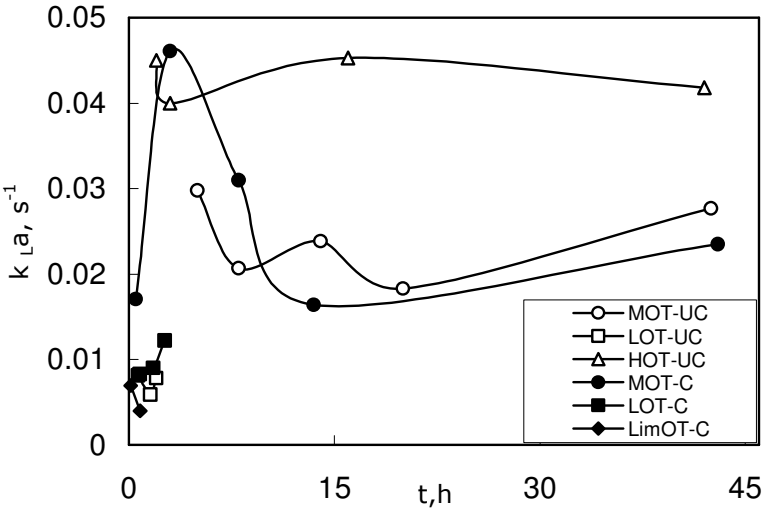


Figure 4.20 The variations in K_La profiles with the cultivation time, air inlet rate, agitation rate and pH strategy

4.4.8.2 Comparison of a thermophilic and mesophilic bioprocess in terms of overall volumetric mass transfer coefficient, K_La

At all bioreactor conditions, average Damköhler numbers of the bioprocesses were found to be high meaning that mass transfer resistances were more effective than biochemical reaction limitations. In order to understand if the mass transfer limitations were due to low oxygen transfer rate that might be caused by the high temperature of the fermentation, the effects of temperature on K_La and OTR were analysed in a system with no reaction in it. Thus a thermophilic and mesophilic bioprocess were compared in terms of overall volumetric mass transfer coefficient, K_La and OTR.

A complete description of the phenomena underlying the oxygen transfer rate in bioreactors depends on many parameters like power input per unit volume, fluid and dispersion rheology, sparger characteristics, coalescence and breakup of bubbles, bubble size distribution, character and intensity of flow of continuous phase (turbulence), relative velocities and path lengths of

bubbles, bubble residence time distribution, dispersed phase hold up fraction, instantaneous and average transfer fluxes (Bailey and Ollis, 1986).

In a roughly manner , oxygen transfer rate (OTR), depends on saturation concentration of oxygen in the medium and the overall mass transfer coefficient, K_La. As the rate limiting step in the transport of oxygen from gas phase into the liquid phase is the liquid-phase diffusion due to the liquid-phase mass transfer resistances, overall mass transfer coefficient, K_La, is assumed to be equal to liquid phase mass transfer coefficient, k_La. Saturation concentration of oxygen in the media depends on temperature and the composition of the medium. As the temperature increases, solubility's of the gases decrease. Thus high temperatures bring an OTR-lowering factor in the media. Composition influences can become complicated when the oxygen can undergo liquid-phase chemical reaction (Bailey, 1986).

The dimensionless groups -Sherwood number ($=K_Ld_b/D_{O2}$), Schmidt number ($=\eta^*/\rho D_{O2}$), and Reynolds number ($=d_b\ u\ \rho/\ \eta^*$)- are often applied to the quantification of overall mass transfer coefficient (K_L) via correlations based on approximate averages of bubble size, superficial velocities and other parameters related to the mass transfer, in bioreactors. K_La depends on the overall mass transfer coefficient, K_L and a. For quantification of K_L , the dimensionless mass transfer coefficient, Sherwood number (Sh) was defined. Sh is a function of only two parameters: Schmidt number (Sc) and Reynold number (Re). Thus apart from bubble diameter and oxygen diffusivity in the media , K_L depends on the dimensionless numbers of Re and Sc. a is the gas-liquid interfacial area per unit volume of the bioreactor (gas+liquid) contents, where head space is not included. a, depends on sparger orifice diameter, volumetric flow rate of the bubbles, number of orifices, diameter of the bubbles, volume of the liquid phase. Bubble formation, bubble breakup and bubble coalescence affect the size of bubbles in bioreactors. Bubble formation depends on the instabilities in the gas stream entering the liquid phase, resulting with breaking of the stream into bubbles, rather than flowing through the vessel as continuous stream. Bubble coalescence depends on the properties of gas-liquid interface; mostly determined by liquid phase solutes. Bubble break up depends on competition between surface tension-stabilizing

the bubble and local fluid forces-tending to tear the bubble apart (Bailey and Ollis, 1986).

At thermophilic fermentations, the temperature is higher than the mesophilic ones. In order to compare mesophilic and thermophilic K_L , a and K_La 37°C and 55°C were chosen to be the operating temperatures of the bioreactor, respectively.As the medium ,used as the production medium, was very similar to water, the rheological properties of the water was used in the calculations.

The following data in Table 4.46, were taken from Perry's chemical Engineering Handbook and Incorpera:

Table 4.46 Properties of water and air

Temperature (°C)	Diffusivity of O_2 in H_2O (m^2/s)	Viscosity of the liquid, η* (centipoise)	Density of the liquid, ρ_L (kg m^{-3})	Surface Tension, σ (N/m)	Density of air, ρ_G (kg m^{-3})
37	3.294×10^{-9}	0.75	993.328	70×10^{-3}	1.128
55	4.753×10^{-9}	0.55	985.696	67×10^{-3}	1.068

At 0.5 vvm and 500 rpm conditions, the following properties are valid for our bioreactor having the characteristics given in Table 4.47:

Table 4.47 Bioreactor Characteristics

Diameter of the bioreactor (D)	121 mm
Height of the bioreactor (H)	270 mm
Orifice Diameter (do)	0.5 mm
Number of Orifice (no)	14
Impeller Diameter (D_I)	53 mm
Liquid volume (V_L)	1.66 L
Gas flow rate (Q)	0.830 L/min
Number of six-bladed impellers (ni)	2

a) Estimation of power input to the bioreactor

Firstly power input at ungassed condition for a single impeller were calculated:

$$P^{degassed}_{single\ impeller} = N_P \rho_L N^3 D_I^5 \quad (W)$$

The Reynolds number for the stirrer, $Re_S = \rho_L N D^2_I / \eta^*$ was calculated to find N_P. Having found the Re_S , N_P the power number, was found to be 5 for both temperatures.

As the bioreactor has 2 impellers (n_i=2),

$$P^{degassed} = P_{single\ impeller}\ n_i \qquad \text{(Nielsen and Villadsen, 1981)}$$

For the gassed power, the following correlation was given:

$$\text{Log } (P_G/P^{degassed}) = -192(D_I\ D^{-1})^{4.38} Re^{0.115} Fr^{1.95DI/D} Ae \qquad \text{(Rose, 1984)}$$

Where $Re = D^2_I N_\rho (\eta^*)^{-1}$

$Fr = D_I N^2 g^{-1}$

$Ae = Q(\ D^3_I N)^{-1}$

From the above equations , the following gassed-power values were found for the bioreactor at 0.5 vvm and 500 rpm and given in Table 4.48 .

Table 4.48 Variation of Power wrt. Temperature

Temperature (°C)	Power (P), W	(P/V_L), W/m^3
37	1.98	1192.77
55	1.96	1180.72

(b) Finding k_L values:

With the assumption of $d_{bubble}=d_b>2.5$ mm & Re>1 for bubbles and with the abbreviation of b for the bubble,

$Sh_b = 1.13\ Sc_b^{0.5}\ Re_b^{0.5}$ was given (Nielsen, 1984) (4.1)

$Sh_b = k_L\ d_b\ D^{-1}_{O2}$ (4.2)

$Sc_b = \eta^*(\rho_L\ D_{O2})^{-1}$ (4.3)

$Re_b = d_b\ u_b\ \rho_L\ (\eta^*)^{-1}$ (4.4)

Putting 4.2, 4.3, 4.4 into 4.1, the following equation was obtained:

$k_L=1.13(D_{O2}\ u_b\ (d_b)^{-1})^{0.5}$ (4.5)

For a rough estimation of k_L in our bioreactor, average values of u_b (bubble velocity) and d_b (bubble diameter) should be found.Because throughout the bioreactor, u_b and d_b change according to both time and height.

(b) Finding the average bubble diameters, d_b

According to Blanch and Clark (1996), there are two regions in the bioreactors. In the first region, bubble size is equal to d_{BO} (the diameter of the bubble determined by the size generated at the orifice). In the second region, bubble size is equal to d_{Be} as the result of two competitive phenomena: coalescence and break up of the bubbles. In this study it was assumed that average bubble diameter is the arithmetic mean of d_{BO} and d_{Be}.Thus

$$d_b = 0.5[d_{BO}+ d_{Be}]= 0.5\left(\left(\frac{6\sigma d_O}{g(\rho_L-\rho_G)}\right)^{1/3} + \frac{1.4\sigma^{0.6}}{\left(\frac{P}{V_L}\right)^{0.4}\rho_L^{0.2}} \right) \quad (4.6)$$

(b) Finding the average bubble velocity throughout the bioreactor, u_b

For bubbles with high Re numbers, the following equation that is valid for low viscosity fluids was used to determine the rise velocity (Moo-Young and Blanch, 1981):

$$u_b = \left(\frac{0.002\sigma}{d_b} + \frac{g\, d_b}{2} \right)^{0.5} \qquad (4.7)$$

As the bioreactor is 27 cm in height and the liquid height is 19 cm, average velocity of the bubble is taken as the bubble rise velocity.

(b) In order to compare the K_La at 37 and 55°C, interfacial area, a was determined according to the following equation:

$$a = \frac{Total\ Interfacial\ area}{Liquid\ volume} \qquad (4.8)$$

$a = \frac{a_d}{(1-\varepsilon)}$ where ε is the gas holdup in the dispersion. (4.9)

Also $a_d = \frac{6\,\varepsilon\,\rho_L^{0.2}}{15\,\sigma^{0.6}} \left(\frac{P_g}{V_L}\right)^{0.4}$ (Moo-Young and Blanch, 1981) (4.10)

Taking ε=0.2,

The parameters are given in Table 4.49.

Table 4.49 Variation of bubble and mass transfer properties with temperature

T, °C	u_B, m/s	d_b, cm	k_L*10^5, s^{-1}	a m^{-1}	k_La, s^{-1}	Re_b	k_La_O, s^{-1} exp.	Max OTR= $C^*_{DO}k_La$
37	0.239	0.349	54	26.7	0.0144	1105	0.0075 (Yilgor)	0.003
55	0.236	0.343	65	27.2	0.0176	1451	0.0069	0.003

The Reynolds number of the bubbles ensure the assumptions of high Reynolds numbers. Also the diameters of the bubbles is larger than 2.5 mm. Thus the starting assumptions are true.

The analysis was carried in a bioreactor with no any kind of reaction in it. As the temperature became higher, average velocity and diameter of the bubbles did not change considerably: Bubble diameter and velocity decreased slightly. However, at higher temperature both the overall mass transfer coefficient and specific interfacial area were higher. Because k_L also depends on diffusivity of oxygen, D_{O2}, other than bubble diameter and velocity. The change in D_{O2}, a parameter increasing with temperature, was more effective on k_L than the changes in u_b and d_b . Thus k_La, which is the multiplication of k_L and a, was higher at higher temperature. Also as T increased, turbulence of the bubbles also increased as expected. Although k_La was higher, OTR were equal at both temperatures. This was because of the higher solubility of oxygen at the lower temperature. Consequently OTR was found to be inaffected by temperature. Thus the mass transfer resistances found in the fermentation broth were concluded not to be due to the high temperature used in the fermentation.

The theoretically found k_La values were approximately 2 folds of experimental physical k_La values because of the assumptions used in the calculations. During comparison of k_La at 2 different temperatures, average values of

surface tension, viscosity, bubble diameter , bubble velocity and specific interfacial area were estimated using some correlations. However the average values of those parameters and the assumptions used may not give the real phenomena, besides the fact that those parameters are very hard to measure. Thus correlations with easily measurable parameters-like power input , superfacial velocity and temperature- should be used for a better understanding of how mass transfer coefficient changes with temperature in a bioreactor without reactions in it.

In order to learn how biochemical reactions within *Bacillus acidocaldarius* affect k_La and OTR , at least 4 different temperatures should be investigated; which can be 40, 50 , 60, 70 ° C. The variations of k_La values obtained at the same hours-wrt. temperature can give a correlation of k_La wrt. temperature, growth and/or product formation.

CHAPTER 5

CONCLUSION

In this study, the effects of bioprocess operation parameters on aromatic amino acid synthesis performance of *Bacillus acidocaldarius* were investigated. For this purpose, the production medium was designed in terms of its carbon and nitrogen sources, in order to achieve a higher cell concentration at the first step. Thereafter, by using the designed medium, the effects of bioprocess operation parameters, i.e., pH and temperature, on aromatic amino acid synthesis performance were investigated in laboratory scale bioreactors. Finally, using the optimum bioprocess parameters obtained in the previous steps, the fermentation and oxygen transfer characteristics of the bioprocess were investigated in pilot scale bioreactors.

5A. Shake-Bioreactor Experiments

The following conclusions were drawn for the shake bioreactors:

1a. The medium design studies for an optimum defined medium started with the following medium: the defined medium for alkaline protease production (Çalık et al., 2003a) that contained (kg m^{-3}) : glucose, 8; $(NH_4)_2HPO_4$, 4.7; $CaCl_2$, 0.2; KH_2PO_4, 2; $NaH_2PO_4.2H_2O$, 7.318; Na_2HPO_4, 0.0438; $Mg(CH_3COO)_2.4H_2O$, 87×10^{-3} (Ref.medium)

2a. Effects of the initial pH were investigated between pH=3-5 in the Ref. medium. The pH of the media were adjusted by sulphuric acid. The optimum initial pH for growth was found 5. Cell concentration was measured at t=17

h and C_X = 0.280 kg m^{-3} at pH_0 =5. No cell growth was seen with the medium having an initial pH of 3.

3a. The effects of $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, NH_4Cl on cell growth were investigated and the initial concentrations were (kg m^{-3}): NH_4Cl, 1.9; $(NH_4)_2SO_4$, 4.707; $(NH_4)_2HPO_4$, 4.71. $(NH_4)_2HPO_4$ was found to be the best nitrogen source for the medium in terms of cell growth.

4a. By using the Ref-1 medium effects of $(NH_4)_2HPO_4$ concentration were investigated at $C_{(NH4)2HPO4}$ = 3, 4, 4.5, 5, 5.5, 6, and 7 kg m^{-3}. As it is seen in Figure 4.2, best cell concentration is at $C_{(NH4)2HPO4}$ = 5 kg m^{-3}. Thus, the medium turned to the following (kg m^{-3}): glucose, 8; $(NH_4)_2HPO_4$, 5; $CaCl_2$, 0.2; KH_2PO_4, 2; $NaH_2PO_4.2H_2O$, 7.318; Na_2HPO_4, 0.0438; $Mg(CH_3COO)_2.4H_2O$, 87×10^{-3} with pH_0 =5, adjusted by H_2SO_4.

5a. In order to adjust the pH of the medium, effects of H_2SO_4, H_3PO_4 and HCl were investigated. The highest cell concentration was obtained with H_3PO_4. Therefore, for the pH adjustment H_3PO_4 was selected, for future experiments.

6a. Effect of the addition of salts was investigated by using the salt solution used in beta lactamase production (Çelik, and Çalık, 2004). Salt solution contained (kg m^{-3}): 250, $MgSO_4.7H_2O$; 1 , $FeSO_4.7H_2O$; 1, $ZnSO_4.7H_2O$; 7.5 $\times10^{-2}$ $MnSO_4.H_2O$; 10^{-2}, $CuSO_4.5H_2O$. With the 33 µl addition of salt solution the cell concentration increased to C_X =0.60 kg m^{-3} from 0.30 kg m^{-3} . The result showed that the addition of inorganic ions to the medium was vital for growth of the bacteria. New medium was named as glucose based defined (GBD) medium and contained (kg m^{-3}): glucose, 8; $(NH_4)_2HPO_4$, 5; $CaCl_2$, 0.2; KH_2PO_4, 2; $NaH_2PO_4.2H_2O$, 7.318; Na_2HPO_4, 0.0438; $Mg(CH_3COO)_2.4H_2O$, 87×10^{-3} ; 0.25 , $MgSO_4.7H_2O$; 10^{-3}, $FeSO_4.7H_2O$; 10^{-3}, $ZnSO_4.7H_2O$; 7.5 $\times10^{-5}$ $MnSO_4.H_2O$; 10^{-5}, $CuSO_4.5H_2O$ with pH_0 =5, adjusted by H_3PO_4 .

7a. The variations in cell concentration utilizing different carbon sources, i.e., glucose, fructose, citric acid, sucrose and maltose, were investigated. Use of fructose as the carbon source resulted the highest cell concentration. Therefore, the effects of initial fructose concentration were investigated at C_F^o =6, 8, 10, 15, 20, 30 kg m^{-3}. At C_F^o =6-15 kg m^{-3} cell concentration were not

affected; however, higher initial fructose concentrations inhibited cell formation. C_F^o =8 kg m^{-3} was chosen, although higher or lower concentrations did not bring significant differences on biomass concentration . The medium containing (kg m^{-3}): fructose, 8; $(NH_4)_2HPO_4$, 5; $CaCl_2$, 0.2; KH_2PO_4, 2; $NaH_2PO_4.2H_2O$, 7.318; Na_2HPO_4, 0.0438; $Mg(CH_3COO)_2.4H_2O$, 87×10^{-3} ; 0.25 , $MgSO_4.7H_2O$; 10^{-3}, $FeSO_4.7H_2O$; 10^{-3}, $ZnSO_4.7H_2O$; 7.5 $\times10^{-5}$ $MnSO_4.H_2O$; 10^{-5} , $CuSO_4.5H_2O$ with pH_0 =5, adjusted by H_3PO_4 was used at further medium design experiments. This medium was called as fructose based defined medium (FDM). The use of salt solution with the carbon source fructose increased cell concentration similar to glucose. The highest cell concentration were obtained respectively as C_X=0.92 kg m^{-3} in the medium containing (kg m^{-3}): fructose, 8; $(NH_4)_2HPO_4$, 5; $CaCl_2$, 0.2; KH_2PO_4, 2; $NaH_2PO_4.2H_2O$, 7.318; Na_2HPO_4, 0.0438; $Mg(CH_3COO)_2.4H_2O$, 87×10^{-3} ; 0.5 , $MgSO_4.7H_2O$; 2×10^{-3}, $FeSO_4.7H_2O$; 2×10^{-3}, $ZnSO_4.7H_2O$; 15 $\times10^{-}$ $MnSO_4.H_2O$; 2×10^{-5}, $CuSO_4.5H_2O$ with pH_0 =5, adjusted by H_3PO_4 .

8a. Effects of supplementing extra $MgSO_4.7H_2O$ into the previous medium were investigated. The cell concentration was not so much influenced by the addition of $MgSO_4.7H_2O$. However $C^o_{MgSO4.7H2O}$ = 1.0 kg m^{-3} was chosen . Thus the medium became the following (kg m^{-3}): fructose, 8; $(NH_4)_2HPO_4$, 5; $CaCl_2$, 0.2; KH_2PO_4, 2; $NaH_2PO_4.2H_2O$, 7.318; Na_2HPO_4, 0.0438; $Mg(CH_3COO)_2.4H_2O$, 87×10^{-3} ; 1.0 , $MgSO_4.7H_2O$; 2×10^{-3}, $FeSO_4.7H_2O$; 2×10^{-3}, $ZnSO_4.7H_2O$; 15 $\times10^{-5}$ $MnSO_4.H_2O$; 2×10^{-5}, $CuSO_4.5H_2O$ [with pH_0 =5, adjusted by H_3PO_4 . With this optimum medium, biomass concentration at 17. h of the bioprocess was obtained as 0.89 kg m^{-3} which was 3.2 –fold higher than the initial reference medium.

9a. Effects of temperature at T= 50, 55, 60 °C and initial pH at pH_o= 3, 3.5, 4, 4.5, 5, 5.5 were investigated in shake-bioreactors. For 50 & 55 °C, optimum initial pH for Cx was 5 while for 60 °C it decreased to 4.5 The highest cell concentration was obtained at 55 °C and initial pH of 5.

10a. In shake-bioreactor experiment, after t>0, only the following amino acids Asn, Ser, Cys, Phe and Trp were found in the fermentation broth. On the other hand, His, Arg, Thr, Ala, Pro, Val, Iso+Leu were not observed in the

fermentation broth throughout the bioprocess. Total concentration of the aminoacids was maximum as 0.24 kg m^{-3} at t=17.5 h of the bioprocess.

11a. In shake-bioreactor experiments tryptophan production was growth associated and the highest tryptophan concentration was obtained as 0.204 kg m^{-3} at t= 17.5 h of the bioprocess. Besides tryptophan, phenylalanine at a maximum concentration of 0.0106 kg m^{-3} was obtained at t= 40 h of the bioprocess.

12a. In shake-bioreactor experiment, acetic acid, succinic acid, α-ketoglutarate, lactic acid, butyric acid and gluconic acid were available in the fermentation broth. Butyric acid only existed at shake-bioreactor experiment, not at any of the bioreactor experiments.

5B. Bioreactor Experiments

Using the designed defined medium the effects of bioreactor operation conditions were investigated and the following conclusions were drawn:

1b. Among the controlled-pH and uncontrolled-pH operations, the highest Trp production was attained at LimOT-pH_C condition as 0.32 kg m^{-3} at t=17 h of the bioprocess where the lowest cell concentration was obtained. On the other hand, the lowest Trp production was obtained at MOT-pH_{UC} .The second highest Trp production, 0.30 kg m^{-3} , was also obtained at LOT-pH_C at t=17 h of the bioprocess.

2b. Among the controlled-pH operations (LimOT-pH_C , LOT-pH_C , MOT-pH_C) with the increase in agitation/aeration rate Trp production decreased. The lowest Trp production was obtained at MOT-pH_C as 0.193 kg m^{-3} at t=13.5 h of the bioprocess, while the highest Trp production was at LimOT-pH_C condition. Among the controlled-pH operations with the increase of oxygen in the medium, Trp production decreased; however when the bacteria used more fructose higher amount of cell were generated. Thus at controlled-pH conditions, with the increase in oxygen, the carbon flux flowed more to biomass synthesis pathway, rather than aromatic amino acid synthesis pathway . In contrast to controlled-pH operations, at uncontrolled-pH

operations, the cell concentration and fructose consumption decreased with the increase in agitation rate.

3b. Among uncontrolled-pH operations, the highest Trp was produced at LOT-pH_{UC} as 0.224 kg m^{-3} at t= 10 h of the bioprocess. At this condition, fructose consumption and cell concentration were also the highest. At HOT-pH_{UC} similar to LOT-pH_{UC}, 0.224 kg m^{-3} Trp was produced but with a 35 h shift in the cultivation time. At this condition, cell concentration and fructose consumption were the lowest. The lowest Trp production was at MOT-pH_{UC} as 0.134 kg m^{-3} at t=45 h of the bioprocess. In terms of productivity, best condition was LOT and the worst condition was MOT. Among uncontrolled-pH conditions, only at LOT-pH_{UC} , maximum Trp production, maximum fructose consumption and maximum cell concentration were obtained simultaneously.

4b. At both MOT and LOT conditions, pH-control produced higher amounts of Trp than the uncontrolled conditions.

5b. The highest tryptophan production rate and the lowest cell concentration were obtained at LimOT-pH_C; namely at the lowest oxygen transfer condition. This result relating low oxygen transfer rate with highest amount of tryptophan is meaningful as aromatic amino acid synthesis pathway is associated with glycolysis and non-oxidative-pentose-phosphate pathway. However cell concentration should also be improved, besides having higher Trp.

6b. Trp is the aromatic amino acid that is produced at the highest concentration, with the highest frequency. Phe production is at a lower amount and frequency than Trp production. Tyr production is very rare, among all the aromatic amino acids produced Tyr was produced only at MOT-pH_C condition, with a maximum concentration of 0.002 kg m^{-3} at t=45 h of the bioprocess. However, Phe was produced at all conditions and at a higher concentration than Tyr. Among all the bioreactor experiment, maximum Phe concentration was observed as 0.018 kg m^{-3} at t=40, at MOT-pH_C condition; while minimum Phe concentration was observed as 0.0033 and 0.0032 kg m^{-3} at t=12, and t=3 h of LOT-pH_C and LimOT-pH_C conditions respectively.

At controlled pH, more Phe was produced at MOT condition. However, at LOT condition more Phe was produced at controlled pH.

7b. At t=0 h, for all bioreactor conditions, the following amino acids existed in the fermentation broth: Asn, Asp, Glu, Gly, Tyr, Cys, Phe, Trp; coming from the precultivation medium. In all controlled-pH operations, Tyr coming from the precultivation was depleted after t=0 h. Also in all of the controlled-pH conditions Asn, Phe, Trp were exist in the fermentation broths. However Arg, Thr, Ala, Pro, Tyr, Val were not excreted into the fermentation broths all the controlled-pH conditions. At MOT-pH_C Asp, Glu, Gly, Tyr ;at LOT-pH_C Asp, Tyr and Cys; at LimOT-pH_C Tyr and Cys coming from the precultivation medium were depleted after t=0 h. His and Cys; Glu; and Asp, Gln, Ser were present in the fermentation broth respectively at MOT-pH_C, LOT-pH_C and LimOT-pH_C. At the cultivation time that corresponds to the time in which the highest Trp obtained, the highest ΣC_{AA} was obtained at LimOT-pH_C as 0.36 kg m^{-3} at t=24 h and the lowest ΣC_{AA} was obtained at MOT-pH_C as 0.22 kg m^{-3} at t=17 h. As the oxygen in the medium increased, obtained maximum ΣC_{AA} decreased.

In all uncontrolled-pH conditions Asp, Gln, His, Arg, Thr, Ala, Pro, Val were not in the medium at all during the bioprocesses. Glu, Tyr and Iso+Leu were exist in the broth only at MOT-pH_{UC} . Maximum ΣC_{AA} was obtained at HOT-pH_{UC} as 0.27 kg m^{-3} at t=45 h. Minimum ΣC_{AA} was obtained at MOT--pH_{UC} as 0.154 kg m^{-3} at t=45 h. Maximum ΣC_{AA} was at HOT-pH_{UC} . At MOT condition, maximum ΣC_{AA} is higher for controlled case than the uncontrolled one. At LOT condition, maximum ΣC_{AA} is also higher for controlled case than the uncontrolled one. pH control increased maximum ΣC_{AA} in the fermentation broth.

8b. At t=0, for all bioreactor conditions, only acetic acid existed in the fermentation broth; coming from the precultivation medium . Acetic acid existed at all operations . Gluconic acid only existed at MOT-pH_{UC} . α-ketoglutarate only existed at at HOT-pH_{UC} . Maximum ΣC_{OA} was at At LOT-pH_C 0.46 kg m^{-3} at t=17 h of the bioprocess. Minimum ΣC_{OA} was at LOT-pH_{UC} was 0.135 kg m^{-3} at t=10 h of the bioprocess.

Among uncontrolled-pH conditions, maximum ΣC_{OA} increased with increasing agitation rate-or amount of oxygen in the medium in uncontrolled-pH conditions. Also Maximum ΣC_{AA} was at HOT-pH_{UC}.

At MOT and LOT condition, for controlled-pH case, maximum ΣC_{OA} were higher than the uncontrolled one. pH control increased total organic acid concentrations. This situation is valid also for the maximum ΣC_{AA} at controlled-pH conditions.

At MOT condition, acetic acid and succinic acid excreted in both uncontrolled and controlled conditions. However, lactic acid and gluconic acid only existed at uncontrolled-pH cases and glutamic acid existed only at controlled-pH case. At LOT condition, acetic acid was present at both conditions. However lactic acid was only excreted at controlled-pH condition.

9b. At controlled-pH conditions, the highest and the lowest mo values were obtained at MOT and LimOT, respectively. Also at controlled-pH conditions, the highest and the lowest ms values were obtained respectively at LimOT and MOT. At uncontrolled-pH conditions, the highest mo value was attained at LOT and the lowest at MOT. The highest ms was at MOT and lowest ms was at HOT.In terms of mo & ms, at MOT, pH control caused higher mo and lower ms. Also at LOT, pH control caused higher mo and lower ms.

10b. At MOT condition, controlled-pH operation had higher oxygen uptake rates than the uncontrolled one at beginning of the bioprocess due to the higher metabolic activities. Similarly, at LOT, controlled case had higher oxygen uptake rates.

In controlled-pH operations, at MOT-pH_C where K_La values changed in the interval of 0.016-0.046 . At LOT-pH_C , K_La values were in the range of 0.0083-0.012 . At LimOT-pH_C , K_La values were in the range of 0.0069-0.004. The highest K_La was at MOT-pH_C and the lowest K_La was at $LimOT_C$, as expected.

In uncontrolled-pH operations, maximum K_La increased with increasing agitation rate. The K_La profiles of uncontrolled-pH conditions were variable.

The range of K_La were 0.0059-0.0081 at LOT-pH_{UC} , 0.0183-0.02978 at MOT-pH_{UC} and 0.04-0.045 at HOT-pH_{UC}.

At both MOT and LOT conditions, controlling pH gave higher maximum K_La values than uncontrolled-pH condition.

11b. In all controlled-pH operations, Damköhler number was increasing wrt. time. Thus as the fermentation continued, mass transfer resistances were becoming more effective than biochemical reaction resistances. . At LimOT-pH_C condition for the first 3 hours , at LOT-pH_C for the first 3 hours and at MOT-pH_C for the first 3 h biochemical reaction resistances were more effective than mass transfer resistances. For LimOT-pH_C condition after t=12 h and for LOT-pH_C after t=6 h of the bioprocesses, mass transfer resistances were more effective . At MOT-pH_C between t= 8-14h biochemical reaction resistances and mass transfer resistances were equally effective.

In uncontrolled-pH operations, for the first 2 hours of LOT-pH_{UC} , for the first 8 hours of MOT-pH_{UC} and for the first 16 hours of HOT-pH_{UC} , Damköhler numbers were always smaller than 1, showing that biochemical reaction resistances were more effective. However for MOT-pH_{UC} t=14 h mass-transfer resistances were more effective and for HOT-pH_{UC} t=42 h of the bioprocess, both of the resistances were equally effective.

In all controlled-pH operations, effectiveness factors were 1 at very early hours of the bioprocesses, however by time passed they decreased to lower values. The same thing also occured for uncontrolled-pH cases.

At controlled-pH conditions the enhancement factor (E) was in the range of (1.7-6.8) while at uncontrolled-pH conditions it was in the range of (1.4-4.3). At both LOT and MOT condition, pH control gave a wider E range than uncontrolled-pH condition, due to higher metabolic activities of the microorganism.

5C. Future Work

For future work the following bioreactor experiments can be done;

1c. At the first step, effect of initial pH-3.5,4,4.5,5,5.5,6 on Trp production should be investigated.

2c. Effect of Temperature on Trp production should be investigated.

3c. 1c should be repeated based on the result of 2c.

4c. Then, effect of controlling pH on Trp production should be investigated at pH=4, 4.5, 5, 5.5 and basing on the results of 3c.

5c. Among all bioreactor conditions, LimOT-pH_C gave highest Trp and lowest cell production while MOT-pH_C gave highest cell concentration . At controlled-pH operations, Trp production was inversely proportional with cell production and amount of oxygen in the environment. Thus Trp production needed lower oxygen while growth needed oxygen at controlled-pH operations. And as Trp was generally growth associated, it is hard to maintain both high Cx and high C_{TRP} at the same time at controlled-pH operations. However at uncontrolled-pH operations, both maximum Trp and maximum cell concentration were attained at the same time, at LOT-pH_{UC} although Trp production was lower than that of controlled one. Thus at uncontrolled operations, for higher cell growth and Trp at the same time, lower oxygen transfer conditions like LimOT-pH_{UC} can be tried.

6c. In the literature it was said that hydrocarbon substrates promoted Trp production (McKetta,1977). In order to increase both Trp and cell concentrations at the same time, oxygen+hydrocarbon gaseous mixture or natural gaseous can be given to the bacteria atthe best condition obtained

7c. To increase both Trp and cell concentrations simultaneously, for the first 14.5 h applying MOT-pH_C and after t>14.5 h applying LimOT-pH_C can be tried.

The following experiments can be done:

8c. 0.2 vvm+ 250 rpm+pHo=5 uncontrolled

9c. 0.2 vvm+500 , 750 rpm+pHo=5 controlled and uncontrolled

10c. 0, 0.1 and 0.6, 0.7 vvm at 250, 500,750 rpm ,pHo=5 controlled and uncontrolled.

11c. 0.2 vvm+250 rpm+pHo=5 controll for the first 3 hour and then stop feeding the oxygen by making it 0 vvm.

12c. 0.2 vvm+250 rpm+pHo=5 controll for the first 3 hour and then stop feeding the oxygen by making it 0 vvm-untill C_{DO} gets zero. And then increase the agitation rate to 500, to find the sole effect of agitation rate on Trp when it has nothing to do with the dissolved oxygen.

13c. 0 vvm+250 rpm+pHo=5 controll for the first 3 or hour and then when C_{DO} gets zero ,increasing the agitation rate to 500 rpm, with still 0 vvm.

14c. Changing the temperature of the medium after a certain hour of the bioprocess.

15c. Complex medium design with a medium having many metalic ions and or developing the already made defined medium: investigating effects of adding indole, antranilic acid, hydrocarbons.

16c. Investigating the effects of adding Tyr, Phe and Trp seperately and/or together to the medium.

17c. The incubation of the solid on the medium should be 24 h, instead of 12 h.

18c. Precultivation medium development and redesign.

19c. Already existing precultivation medium should be investigated to find its Trp profile as the first step of a semi-defined medium design.

20c. Genetic manipualtions with the bacteria for overproduction of Trp.

21c. Using only-Trp-absorbing zeolites in the media of fermentation to avoid feed-back inhibition or repression of the product, or continous production of Trp to avoid those problems.

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APPENDIX A

Calibration of *Bacillus licheniformis* Concentration

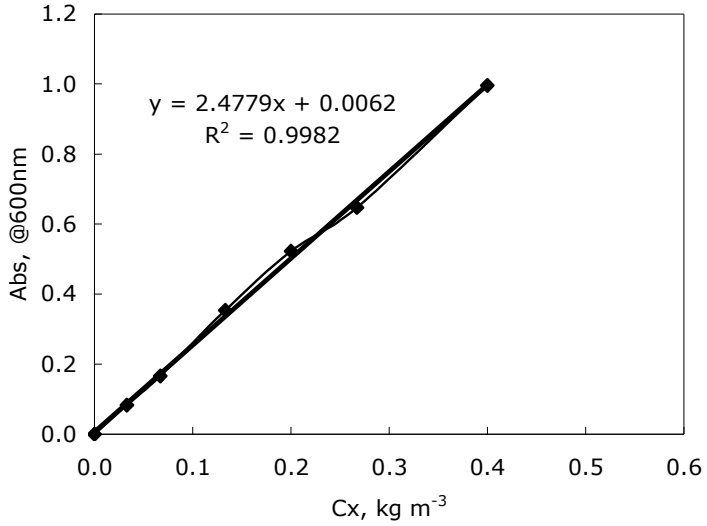

Figure A.1 Calibration curve for *Bacillus licheniformis* concentration

Slope of the calibration curve, m=2.49 1/kg m^{-3} (λ=600 nm)

$$Cx = \frac{Absorbance}{2.49} \times DilutionRate$$

APPENDIX B

Calibration of Reduced Sugar Concentration

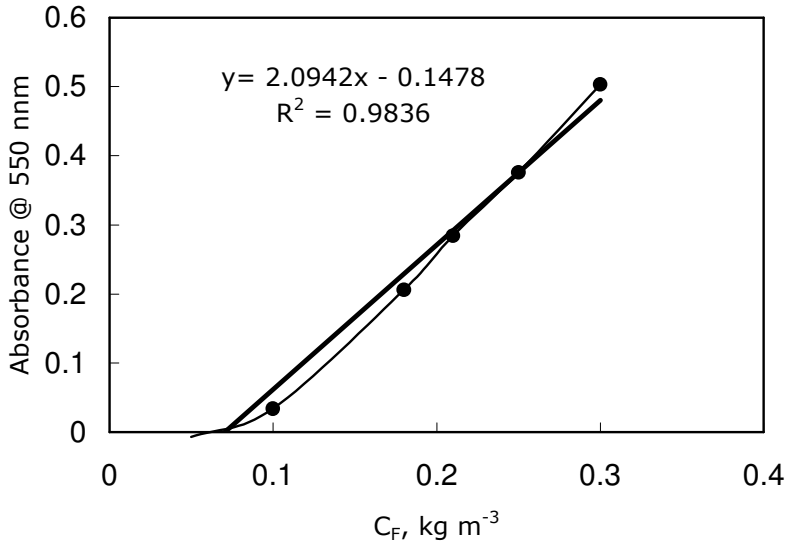

Figure A.3 Calibration Curve of the DNS solution

$$C_F = \left(\frac{Absorbance + 0.1478}{2.0942} \right) \times DilutionRate$$

APPENDIX C

Preparation of DNS Solution

1. a) 880 cm^3 of 1 % (m/v) DNS solution is prepared by dissolving 8.8 g dinitrosalisilyc acid in 880 cm^3 distilled water.
b) After addition of 225 g ROCHELLE salt (sodium potassium tartarate), the solution is mixed.
c) 300 cm^3 of 4.5 % NaOH, prepared by dissolving 13.5 g NaOH in 300 cm^3 distilled water, is added to this solution.
2. a) 22 cm^3 10 % NaOH, is prepared by dissolving 2.2 g NaOH in 22 cm^3 distilled water.
b) 10 g christalized phenol and 100 cm^3 distilled water are added to the solution.
c) 60 cm^3 is taken from this alkali-phenol mixture, 6.9 g $NaHCO_3$ is added and mixed.

The solution obtained from the first step is mixed with that from the second step and then they are stirred until ROCHELLE salt is dissolved. The prepared solution is kept in dark-colored bottle at 4°C and it should be used after 48 h.