



Optimization of Operating Conditions of Increasing HBsAg Protein Expression in FED BATCH Fermentation Process by Changing *Pichia Pastoris* Culture Medium Conditions and Examining Growth of Yeast Cells by Methanol Testing

Payam Moradi Zalam Abadi ¹, Alireza Fazlali ², Seyed Nezamedin Hosseini ³, Ehsan Jafarbeigi ^{1,4}, Farhad Salimi ^{*1}

¹ Department of Chemical Engineering, Kermanshah Branch, Islamic Azad University, kermanshah, Iran

² Department of Chemical Engineering, Faculty of Engineering, Arak University, Arak, Iran

³ Production & Research Complex, Pasteur Institute of Iran, Tehran, Iran

⁴ Young Researchers and Elite Club, Kermanshah Branch, Islamic Azad University, kermanshah, Iran

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Abstract

Optimization of the culture medium and induction conditions in the fed-batch fermentor is the commonest and easiest method to increase the overall productivity of recombinant proteins production. Environmental conditions such as temperature, dissolved oxygen (DO), PH, and agitation also have a major impact on the expression of recombinant protein. Since the production of the recombinant protein in *pichia pastoris* is highly affected by induction conditions, providing induction conditions is one of the most important ways to increase the productivity rate. AOX1 gene enables recombinant proteins expression at the highest level in methylotrophic yeasts. This gene is activated by methanol and induced protein expression. Low and high methanol amounts respectively lead to its reduced induction ability and overproduction of formaldehyde and other toxic substances. However, given that carbon is considered as microorganisms feed, therefore, the injection of pure methanol in a timely manner in sufficient quantities in specified time intervals, the necessary amounts of carbon were supplied for feeding them. Also, vitamins such as vitamin A and B were regularly injected to the extent necessary in the fermentation process. Therefore, this study aimed to investigate the methanol feeding process, an increase in specific growth rate (μ), OD and dry weight (WW), comparison of the increase in OD and WW. The results showed that the performance of the feeding profile is improved as much as possible according to the existing facilities.

Keywords: *Pichia pastoris*; Methanol utilization pathway; Fed-batch fermentation; Recombinant protein expression; Genome annotation

1 Introduction

Hepatitis B virus (HBV) is the leading cause of cirrhosis and liver cancer and HBV infection is now a global problem (1-4). In a research on various serum proteins in 1963, Bloomberg accidentally discovered an unknown protein in the blood of an Australian native. This protein was introduced as Australian (AU) antigen and it was found that this protein is related to HBV. In 1968, Prince Okohi and Murakami found that AU antigen existed in the blood of people infected with hepatitis B only in the same year. Bloomberg could isolate some amount of hepatitis B surface antigen (HBsAg) in the blood of an infected person and continued research revealed that this antigen is the main symptom for the incidence of this disease. Dane et al.

(1970) could observe this virus in the blood of those suffering from this disease. Continuous experiments showed that HBsAg can stimulate the body's protective system to secrete antibodies. In 1971, Krugman discovered that the injection of blood infected with killed HBV can stimulate hepatitis B antibody (5). It was also proposed that nucleic acid fragments separated by Bloomberg have the ability to stimulate the immune system against this virus (6). Blumberg and Millman provided ground for the development and advancement in making the vaccine, in which HBsAg can initiate the body's protective response to this disease. So, they proposed that this vaccine to be prepared from the antigen in the blood of infected people. The strategy for vaccine design was first to use the blood infected with the weakened virus to stimulate the immune system. But, it soon became apparent that the use of antigen purified from the infected blood is more effective and safer. Although Fox Chase Cancer Centre (FCCC) was awarded the patent for this discovery in 1969, many scientists have carried out extensive

Corresponding Author: Farhad Salimi, Department of Chemical Engineering, Kermanshah Branch, Islamic Azad University, kermanshah, Iran. Email: f.salimi@iauaksh.ac.ir.

researches on this concept and were able to purify it and enable its public use. This vaccine provided the immunity rate of more than 90% without causing any side effects (7). Router et al. (1977) proposed making HBV vaccine through recombinant technology. This production process could provide safe abundant product. The first system was carried out by bacterial cells, but experiments yielded no success (8). Before the development of recombinant technology, the hepatitis B vaccine was produced by extraction of hepatitis B surface antigen (HBsAg), the main ingredient of hepatitis B vaccine, from the plasma of infected patients (34, 37). Afterwards, Hall developed this system by yeasts and Valenzuela et al. (1982) (7) managed to achieve the first purified HBsAg product by recombinant genetic system using yeasts. Finally, after nine years of research, the first vaccine was made in 1986 and the US Food and Drug Administration (FDA) issued the permission for its general use. After a short while, French scientists were able to make the same proteins that had the same ability to stimulate the immune system. In this system, the recombinant plasmid was transferred into ovary cells of a kind of Chinese mountain hamster mice. This plasmid was a little different from the yeast plasmid because it contained surplus of HBV genome. This means that in addition to protein S gene, it also contained Pre-S2 protein gene. Although this system was different from the yeast, it contained same 22nm protein fragments that stimulated the immune system.

1.2 *Saccharomyces cerevisiae*

The first recombinant HBV vaccines, which can be produced commercially, were made through genetic manipulation of the yeasts. Normally after being made in eukaryotic cells, proteins often enter the Golgi system to undergo changes in their structure. For this reason, eukaryotic cells such as yeast are used to produce the recombinant HBV vaccine. A variety of yeasts that are used in the production of recombinant HBV vaccine include: *saccharomyces cerevisiae* and *pichia pastoris*. There were certain limitations associated with the use of *saccharomyces cerevisiae* to produce HBV vaccine, because its total protein efficiency (TPE) is 1% to 5%. Also, placing a transgene in the yeast causes tensions in host cells, and the produced foreign protein is considered toxic for *saccharomyces cerevisiae* in many cases. Currently, *P. pastoris* has attracted major attention in the healthcare industry for efficient largescale production of recombinant products (34-36). The first plasmid, in which hepatitis B antigen is produced under the control of alcohol dehydrogenase gene promoter (ADH1), was transformed in *saccharomyces cerevisiae* by Valenzuela et al. This system has yielded an expression rate of about 10-25 $\mu\text{gHBsAg.L-1 culture}$ (8). Later, the expression of this gene reached to about 50 $\mu\text{gHBsAg.L-1 culture}$ in *saccharomyces* under the control of 3-phosphoglycerate kinase gene promoter (PGK) (9). Miyanojara et al. achieved expression rate of 2.8 $\mu\text{gHBsAg.L-1 culture}$ under the control of acid phosphatase gene promoter (10). Under the control of glyceraldehyde-3-phosphate gene promoter (GPD), S gene expression rate reached 3 $\mu\text{gHBsAg.L-1 culture}$ and about 20 culture mgHBsAg.L-1 (6-fold increase) in *saccharomyces cerevisiae* using the batch fermentor and the batch-fed fermentor, respectively (11). The fact that the cell can secrete the protein out of the cells is very important. Chen et al. made a tremendous effort on this issue, but it was useless (9). Due to problems associated with the *saccharomyces cerevisiae* use, researchers used methylotrophic

yeasts. *Pichia pastoris* was first identified as a high-performance system in producing this antigen. In the first experiment, the production rate reached to 48 $\text{mgHBsAg.L-1 culture}$ 1 (12). At the industrial scale, a change in the cell structure and use of alcohol oxidase (AOX) gene promoter reduced the production rate to 1.3 $\text{mgHBsAg.L-1 culture}$. Phillips Co. signed a contract with Salk Institute Biotechnology / Industrial Associates. Inc. (SIBIA) for the development of *pichia pastoris* as a microorganism for the expression of heterologous proteins in 1980. Researchers isolated AOX1 promoter in SIBIA and used it as the promoter gene in manipulated *pichia pastoris*. AOX1 can increase the total extract soluble protein up to 30% and methanol grows merely in *pichia pastoris* (13-15). AOX1 enzyme is responsible for the methanol of the first oxidation reaction in *pichia pastoris*. It is synthesized by AOX1 promoter, which has been widely used in the expression of recombinant proteins in this microorganism (16-18). This expression system is widely used for the expression of heterologous proteins (38). The advantages of *pichia pastoris* compared to the *saccharomyces cerevisiae* yeast include:

1. *Pichia pastoris* has a very active promoter for methanol utilization and its oxidation to formaldehyde and hydrogen peroxide and this activity is carried out by AOX enzyme.

2. The expression of this gene is highly regulated. In other words, when it uses glucose or ethanol as the carbon source, AOX is inactive. But when the yeast is cultured in medium containing methanol, AOX can increase total cell proteins by 3% to 5%. There are 2 AOX genes, including AOX1 and AOX2. AOX2 plays an inhibitory role for the expression.

3. The use of methanol for this yeast leads to creation of a special characteristics compared to other yeasts because *saccharomyces cerevisiae* and most of other yeasts lack this ability. Also, this yeast's ability to grow in the methanol can be used to prevent contamination of the fermentation medium during fermentation. Considering reasons mentioned above, today, *pichia pastoris* has become a more suitable candidate for use in the genetic engineering. To achieve an optimal strategy to achieve the highest amount of recombinant protein production, factors such as temperature, PH, specific growth rate of yeast physiology, culture conditions and feed composition percentage have been investigated. Factors such as temperature have been investigated frequently. *Pichia pastoris* fermentation process is carried out in three ways: 1) Glycerol batch phase 2) Transformation phase (from development to production) 3) Methanol induction phase and the induction phase was investigated among the three above-mentioned phases.

1.3 *Glycerol batch phase*

The purpose of this phase is to obtain the maximum amount of yeast in the shortest time. The highest μ in glycerol (1/h) has been reported to be 0.18(19) that is much higher in comparison with that of methanol (1 / h) (0.14) (20). Usually μ is much less than this amount in methanol phase that is due to the production of protein and negative impact of its metabolic burden on the growth. This phase begins with glycerol concentration of 40g / L independently of the type of *pichia pastoris*. Glycerol with higher concentration acts as an inhibitor. Brierley et al. proposed 6% as the maximum amount for glycerol concentration (21). Scientists measured the ethanol amount as about 2% in glycerol concentration of above 7%. This ethanol concentration is toxic to the yeast. The dry biomass obtained from this amount of substrate is approximately 0.5. The amount of dry weight cells

obtained at the end of this phase is 100g / L. The end of this phase is characterized by a sudden increase in DO, which is due to the end of substrate and lack of oxygen consumption.

1.4 Transfer phase

The biomass amount needed to be increased until achieving high amount of cell mass. This is one of the objectives of this phase to the extent that the presence of glycerol does not prevent methanol from entering the induction phase. Some researchers have added glycerol with a fixed amount to the culture medium and some others have added glycerol exponentially. In this method, the dry weight of cells can be increased even to more than 120g/L. This phase is usually completed with a gradual increase in methanol level. In this method, the cells become ready to enter the induction phase. Different methanol feeding programs have been reported. (a) Gradual increase in methanol in proportion to the DO amount (20). (b) Maintaining the methanol concentration of about 4g / L (22). (c) Inducing 1hour hunger before entering the induction phase to ensure that all the available glycerol is consumed (23). (d) Adopting glycerol reduction program along with fixed glycerol feed rates (24).

1.5 Methanol induction phase

Pichia pastoris starts producing the recombinant protein under the control of AOX promoter in this phase. Specific growth rate (μ) has a major impact on the amount of protein expression, thus feeding must be carried out in such way that we achieve the maximum expression rate. It is very important to control the concentration level because its high levels and cause toxicity and its low levels prevent protein expression. Induction with methanol is a very important phase, because AOX activity is low at the beginning and the methanol accumulates easily. At this stage, the oxygen consumption rate is limited with rapid increase in AOX activity. *Pichia pastoris* strains are sensitive to high methanol concentrations remained in the medium and sudden methanol accumulation decreases AOX activity and leads to cell death. Methanol is controlled using direct or indirect measurements. Methanol direct measurement in this process is an important tool in optimizing feeding strategies. A methanol sensor with silicone tube is commercially available in the market for the direct measurement of methanol (25). Gas analyzer is a unique method that is based on the measurement of oxygen and carbon dioxide leaving the fermentor. In this method, other output gases such as ammonium may create a nuisance, but it can be solved with a modification(26). This device can be used to control methanol concentration feedback and in methanol and temperature-limited fermentation processes. When the necessary equipment is not available for direct control, another strategy is used to adjust the methanol concentration that is called DO spike method. In this feeding method, methanol concentration is adjusted in such way that the methanol steams are prevented from being exited, which is detected through a sudden increase in DO (27). This method has its own defects, too. If an inhibitor stops the process, DO level rises, as a result of which the injected methanol level increases, which in turn leads to methanol accumulation and cell death. Some direct methods are based on the indirect method. For example, in sequential injection analysis, seven samples are taken and analyzed per hour. But the disadvantages of this method include an increase in the likelihood of contamination and yield loss due to continuous sampling (28). Methanol accumulation control is also feasible using indirect method, i.e.

by stopping the feeding process. The most important indirect control devices include Gas chromatography (GC), High Performance Liquid Chromatography (HPLC) and enzymatic reactants. In this method, we can measure the concentration of other substances and even protein. In case of limited methanol concentration, DO response is rapid and methanol feed can be increased. This indirect measurement should be continued until the DO amount reaches the desired minimum level. Then methanol feeding rate is maintained at a constant rate. Sometimes a feedback control loop is used to maintain DO level. But it is practically unusable because the sampling and testing take a long time. Therefore, direct methods are usually used.

1.6 Fermentation

Fermentation is a process that is carried out by using energy released from the oxidation of organic materials such as carbohydrates. In industry, fermentation refers to so-called chemical reactions, the catalyst of which is microorganisms, whether aerobic or anaerobic process.

1.7 Fermentor

Fermentor is a device, in which fermentation operation is carried out. Fermentor used in this research, was Vessel single jacket model fermentor with Winpack brand. Fig 1 shows a view of the fermentor and other parts of it.

1.8 Conventional methanol feeding methods in batch culture

1.8.1 *Pichia pastoris* yeast

Methylotrophic yeast *pichia pastoris* is a suitable host for production of recombinant proteins. One advantage of this host is, in fact, having a very effective regulation promoter called AOX. *Pichia pastoris* uses methanol as a source of carbon and energy. Methanol is also considered as an inducer in this system. AOX enzyme is a catalase that decomposes hydrogen peroxide to water and oxygen. Some of the formaldehyde produced by AOX leave the peroxisome and is oxidized mainly to carbon dioxide and formate. This reaction occurs with cytoplasmic dehydrogenase that provides the energy source for cell growth on methanol. High cell density culture with *pichia pastoris*, AOX promoter enables achieving production of large quantities of recombinant protein. However, due to the accumulation of formaldehyde and hydrogen peroxide in the cell through methanol metabolism, methanol concentration in the fermentor is effective both on cell growth and level of protein secreted by cells. Therefore, proper adjustment of methanol concentration in *pichia pastoris* fermentation is necessary not only to maintain the induction of genes under the control of the AOX promoter, but also to prevent the accumulation of excessive methanol concentration in the medium sites in the centrifuge.

1.8.2 Methanol consumption cycle

Methylotrophic yeasts belong to four species: *Hansenula*, *Pichia*, *Candida*, *tropicalis*, all of which have methanol consumption cycle. The main reactions in this cycle are shown in Fig. 2. To grow in methanol, the yeast is adapted by induction of AOX gene promoter, dihydroxyacetone synthase (DAS) and many different enzymes that can participate in this metabolism (29). In these species, AOX can produce more than 35% of the cellular protein, while this gene is inactive in glucose and glycerol or ethanol. AOX oxidizes methanol in the first step and then converts it to formaldehyde and hydrogen peroxide.

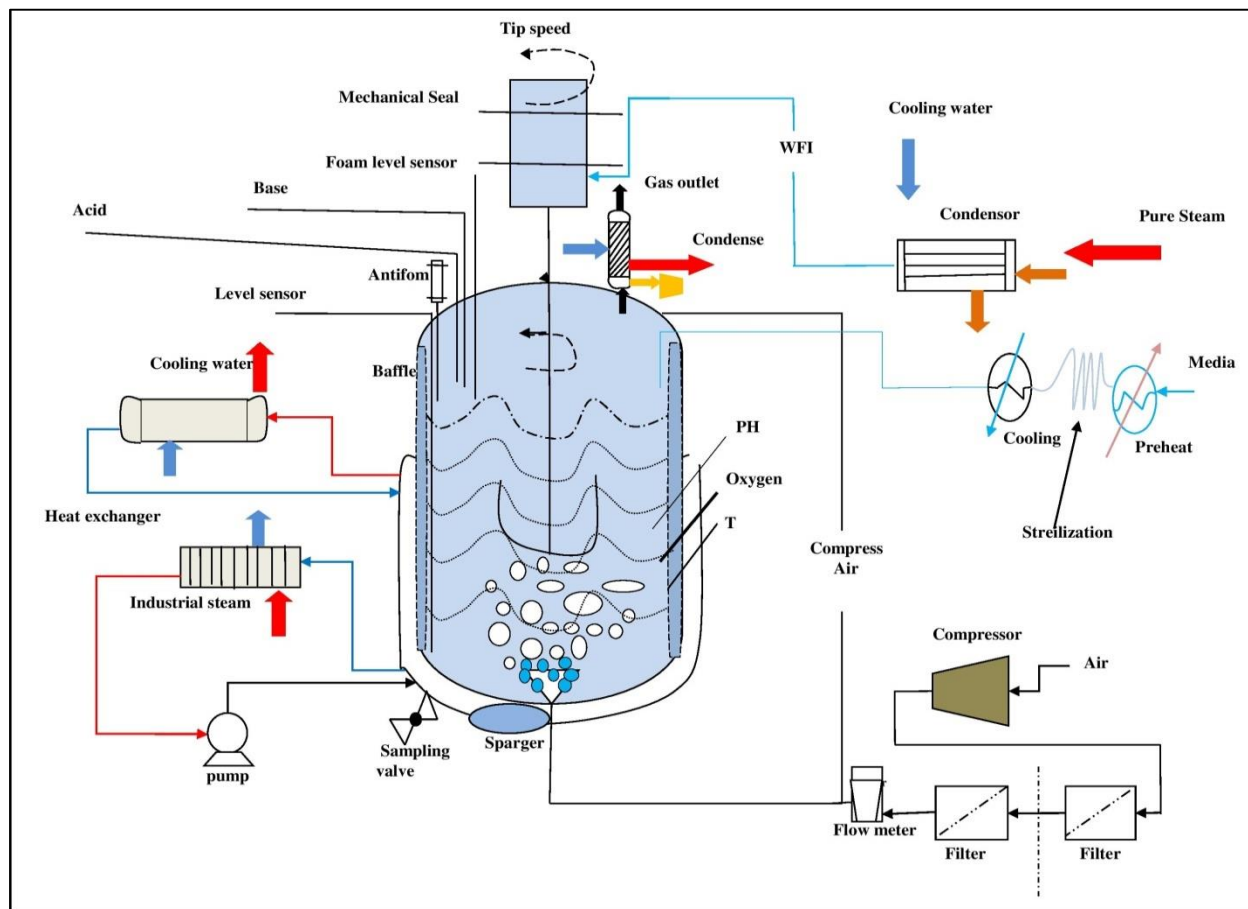


Figure 1: A view of the used fermentor

This reaction is performed in an organ, called peroxisome to prevent the toxicity of hydrogen peroxide in the reaction (30). Peroxisomal catalase breaks down hydrogen peroxide to water and oxygen. Formaldehyde enters the cytosol from peroxisome, where it forms a complex with the regenerated glutathione. Then, it is oxidized and carbon dioxide is produced by the dehydrogenation reaction in two stages. Formaldehyde-GSH complex is converted to formate by formaldehyde dehydrogenase enzyme and formate is oxidized and is converted to carbon dioxide by formate dehydrogenase enzyme. Oxidation in this cycle not only produces energy in the form of NADH, but also plays a very important role in eliminating formaldehyde toxicity in the methylotrophic yeast (31). Formaldehyde can then be re converted to methanol by formaldehyde reductase enzyme. Formaldehyde reductase and alcohol oxidase enzymes create a cycle that controls the cellular amount of formaldehyde and NADH. The residual formaldehyde reacts with xylose -5-phosphate (Xu5P) through transketolase reaction and produces two products of Dihydroxytryptamine acetone (DHA) and glyceraldehyde - 3-phosphate (GAP). This reaction is carried out

by Dihydroxyacetone synthase enzyme. Dihydroxyacetone synthase enters the cytosol and is phosphorylated by Dihydroxyacetone kinase enzyme. It is later converted to fructose-1, 6-bisphosphate (FBP) after reacting with glyceraldehyde-3-phosphate in the aldolase reaction and is finally converted to fructose 6-phosphate (F6P) by 8-phosphate phosphatase. F6P enters the pentose phosphate pathway and reproduces xylose phosphate. During each of three cycles, one glyceraldehyde - 3 - phosphate molecule is produced and the biomass is formed by the standard gluconeogenesis reaction. Fig. 3 shows a simple metabolism shape in methylotrophic yeasts (14). Tri-carboxylic acid cycle (TCA) plays an important role in energy supply for the growth of methylotrophic yeasts.

2 Materials and method

Required materials and equipment have been listed in Tables 1 and 2. Fig. 4 shows the algorithm of how to implement the project.

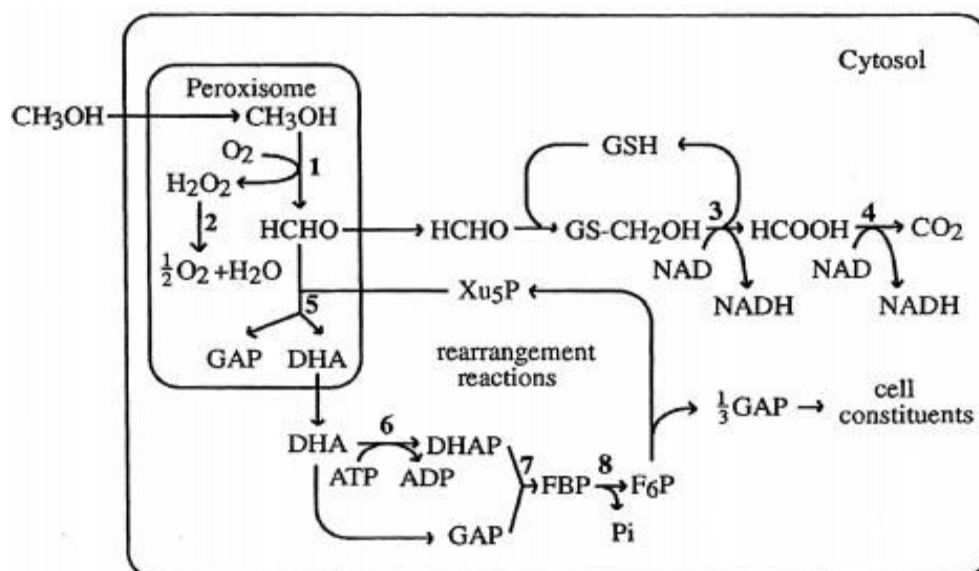
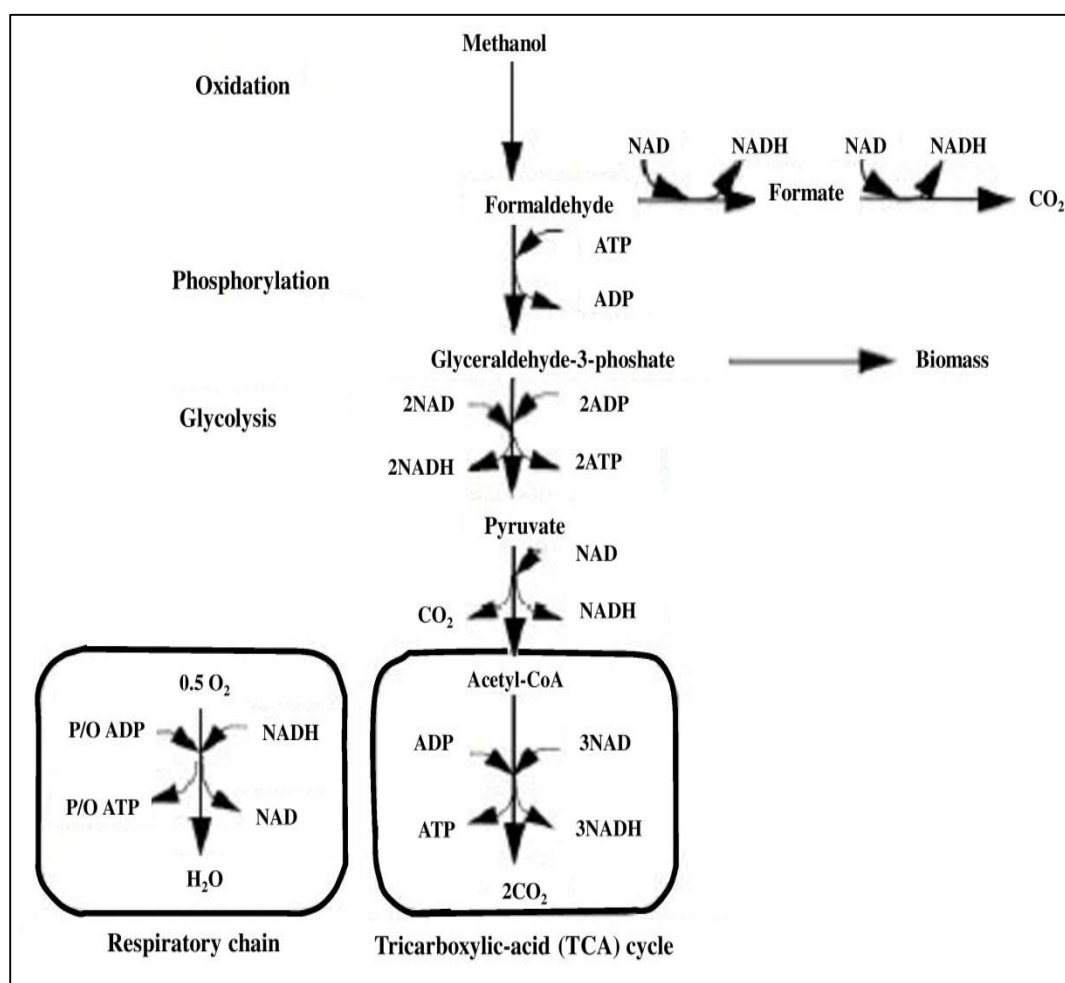


Figure 2: *Pichia pastoris* metabolism cycle in methanol. 1, alcohol oxidase; 2, catalase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase, 5, dihydroxyacetone synthase; 6, dihydroxyacetone kinase; 7, fructose 1,6-biphosphate aldolase; 8, fructose 1,6-bisphosphatase

Figure 3: *Pichia pastoris* simplified metabolism cycle in methanol (14)

2.1 Used materials

In this study deionized water (hardness of less than 1 microsiemens), extract of microbiological yeast, agarose, sodium chloride, calcium chloride dehydrate, methanol, crystal violet, EDTA, glycerol, di-sodium hydrogen phosphate anhydrous, agarose, diethyl barbituric acid, diethylbarbituric acid sodium, caprylic acid, sodium acetate dehydrate, copper sulfate heptahydrate, ammonium sulfate, manganese sulfate heptahydrate, zinc sulfate monohydrate, iron sulfate heptahydrate, potassium chloride, potassium hydrogen phosphate, potassium iodide, acrylamide, bis acrylamide, ammonium sulfate, bromophenol blue, acetic acid monohydrate, standard molecular weight marker, and pichia pastoris yeast have been used.

2.2 Needed equipment

In this study Eppendorph centrifuge model 5804, peristaltic pump model VERDERLAB (2 pcs), Nikone optical microscope, glass beads with a diameter of 0.5 micron, pH meter model Mettler Toledo, Eppendorph spectrophotometer, IKA horizontal stirrer, IKA horizontal shaker, Eppendorph 37 ° C incubator, Eppendorph 30 ° C incubator, Emersan 2 to 8 ° C refrigerator, REVCO -20 ° C freezer, IKA Vortex, microbial hood, elisa reader, glass utensils, magnet, pence, syringes, dowel, millipore filter paper Wharman GB002, WinPact Glass 10 liters fermentor, and oxygen capsule have been employed.

Table 1: Required nutrients and design of medium

Medium	Concentration
(NH ₄) ₂ SO ₄	20.31 g/L
MgSO ₄ ·7H ₂ O	6.92 g/L
K ₂ HPO ₄	16.77 g/L
CaCl ₂ ·2H ₂ O	0.46 g/L
H ₃ PO ₄ %85	8.8 g/L
Trace element	1 g/L
Glycerol	40 g/L
W.F.I	2 lit

2.3 Working method

1. Preparation of 4.5 lit complex medium (including different types of proteins, lipids and carbohydrates)
2. Pouring the medium into the fermentor vessel.
3. Blocking all fermentor container openings by cotton, foil and clamp.
4. Autoclaving the fermentor container along with its contents for 30min.
5. Connecting feed, air, sensors, and temperature and pH probes to the fermentor container after autoclaving under complete sterile conditions.
6. Setting feed, temperature, rotational speed of the blades and pH parameters by fermentor monitor.
7. Inoculating 0.5 lit of inoculated culture medium by siphon and peristaltic pump in sterile conditions
8. Injection of Vitamin A, Vitamin B, Trace Element

9. Beginning the fermentation process and continuing it until inhibition of cell growth.

10. Harvest

11. Biomass centrifugation and collection

Table 2: Required nutrients and design of medium

Vitamin A & B	Concentration
Di-Potassium hydrogen phosphate	4 g/L
Biotin	0.8 g/L
Myo-inositol	8 g/L
Calcium Pantothenate	0.8 g/L
Pyridoxol Hydrochloride	0.8 g/L
Thiamine Hydrochloride	0.8 g/L
Nicotinic Acid	0.2 g/L
W.F.I	1L

2.4 Determining cell dry weights

First, four clean 15 ml falcons, two of which are named A and B are prepared. The falcons are later weighed using a sensitive scale. The yeast mixture is taken to the extent necessary and 5 ml of the mixture is added to each of A and B falcon. Then they will be placed in two opposite Mixtures are later centrifuged at 22 °C for 15 minutes at 3000 rpm. The supernatant is poured into another falcon and kept into the freezer so that it is used later to measure methanol. After being dried, the falcons are weighed again. If we multiply the primary and secondary weight difference by 200, the dry weight will be obtained.

2.5 Expression under the control AOX1 gene promoter

Heterologous proteins are often produced in the *P. pastoris* yeast under the control of very effective AOX1 promoter. This promoter controls the expression of the AOX1 enzyme. For the first time, Ellis et al. (1985) were able to isolate AOX1 gene. *P. pastoris* genome consists of two alcohol oxidase genes (AOX1, AOX2). AOX1 has the greatest responsibility in regulating alcohol oxidase activity in cells. This gene produces high levels of AOX1 enzyme in cells (more than 30%); therefore, this promoter can cause expression of recombinant proteins higher than 12 g / L (32). Considering the ability their methanol consumption rates, yeasts are divided into three sub-groups:

Positive methanol consumption (Mut⁺): Both AOX1 and AOX2 genes are present and have good efficiency and yeast grows well in methanol.

Slow consumption of methanol (Mut^s): AOX1 gene is damaged and AOX2 gene only works, but the resulting slows methanol consumption depends on the poor transcriptional properties of AOX2.

Negative methanol consumption (Mut⁻): Both AOX1 and AOX2 genes are inactive, methanol cannot be metabolized, but its presence is necessary for induction of recombinant proteins expression. The maximum specific growth rates in methanol are 0.14h⁻¹, 0.04h⁻¹ and 0.0 H⁻¹, respectively for Mut⁺, Mut^s and Mut⁻ species of *P. pastoris* yeasts (33). Figures 5, 6, 7, 8 and 9 respectively show methanol feeding process, an increase in μ, OD, dry weight (ww), comparison of an increase in OD and ww.

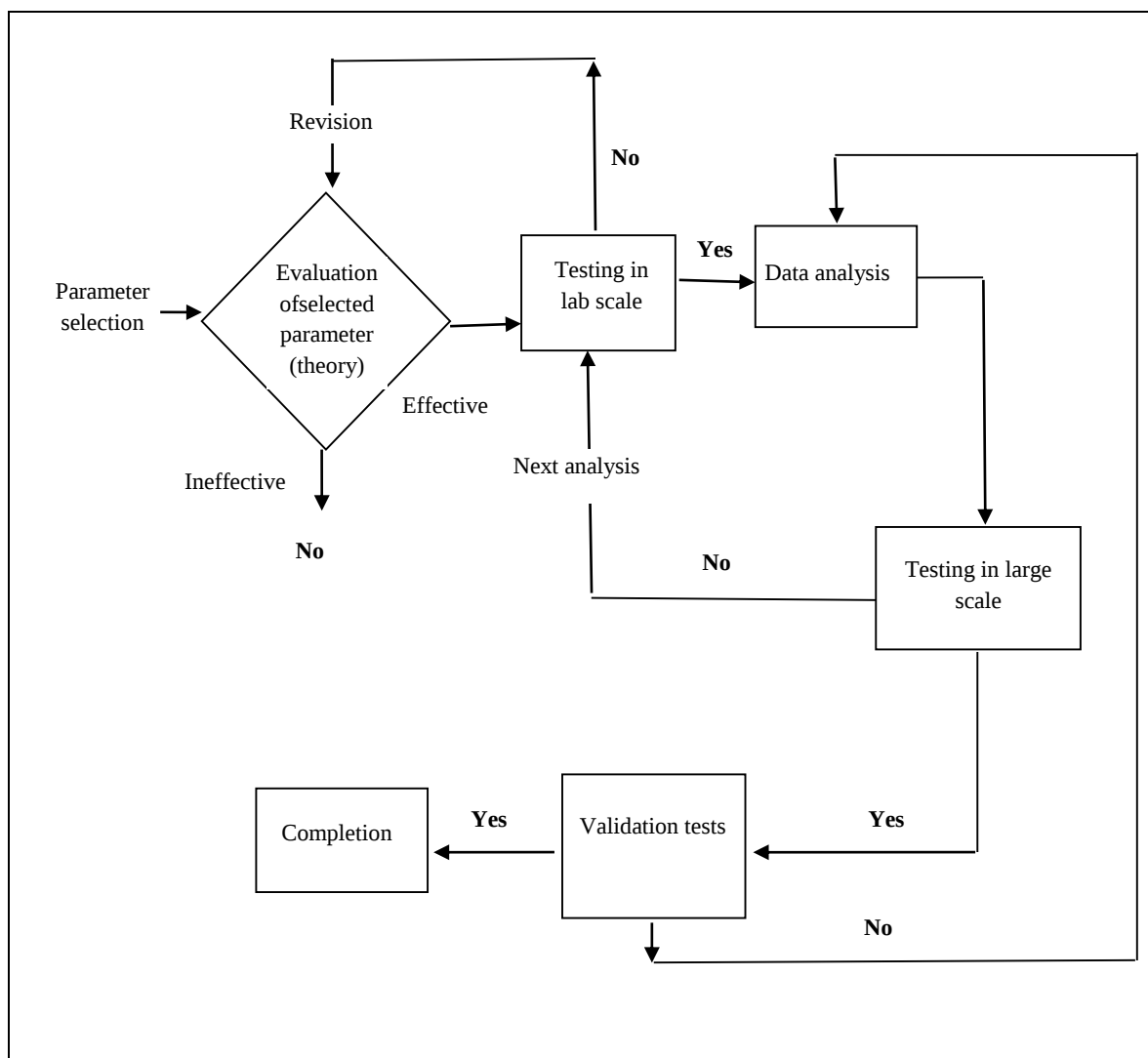


Figure 4: Algorithm of how to implement the project

2.6 Fed-batch culture (the induction phase)

Fed-batch culture is a method of choice to achieve high cell density. Implementing fed batch fermentation process, despite its numerous advantages, due to problems such as restriction or inhibition of raw materials (especially carbon source) required for yeast growth, high-speed production of CO₂, heat, excessive need for oxygen and limited capacity to transfer oxygen, a sharp increase in viscosity and reduced mixing efficiency, plasmid instability and reduced yield in case of prolonged process, product degradation by intracellular proteases, accessory metabolite accumulation to the inhibition level, has always been associated with challenges. Considering its ease of implementation and possibility of changing the growth rate of the cell, the exponential feeding method is a method that is more commonly used for the implementation of high cell density culture of various microorganisms. Research has shown that the specific productivity in high cell density culture is usually less than the "fed-batch culture that is probably due to the fact that most fed-batch processes are implemented under low specific growth rate and the limited substrate level conditions. At this stage of high cell density culture (induction phase) of *Pichia*

pastoris yeast, methanol is considered as an inductor for the expression of recombinant protein and energy source for cells. Accumulation of methanol in the medium is considered as a growth and production inhibitor. On the other hand, its deficiency reduces the growth rate and increases the process time. Each of limited methanol feed rate, fixed-rate and exponential feeding, feeding strategy based on the growth rate, and etc. attempts to improve the production of proteins. The exponentially methanol feeding strategy was used in Pasteur Institute in such a way that the specific growth rate was almost at about fixed rate of 0.016. The medium conditions of the process were almost unchanged and only the amount of input methanol was changed. Medium pH was controlled at 4.5 and since CO₂ gas is produced in this process, the medium pH decreases and ammonia is used to control it. In addition to regulating pH, ammonia is also considered as the nitrogen source. The needed oxygen was first supplied by the compressed air and Due to the impossibility of increasing the pressure in the fermentor and atmospheric nature of the process, oxygen deficiency was evident. Therefore, pure oxygen capsules were used at this stage. Agitation plays a fundamental role in mass

transfer and increasing oxygen time both in terms of creating dispersion and breaking apart the oxygen bubbles. At the beginning of the process, the agitation was set at 250 rpm and finally was set at 600 rpm due to the increased viscosity of the solution. Medium temperature was controlled unchanged at 30 °C. Temperature control system was comprised of a heat exchanger and a pump that circulates the water in the pipe inside the fermentor.

3 Results and discussion

As can be seen in Fig.5, Since the yeast is inadaptible with the change in type of carbon source (carbon source was changed to glycerol source) in the early hours of its growth, Methanol is injected slowly into the fermentor and the increasing trend of methanol consumption rises with a more gentle slope. In this phase, the yeast needs time to metabolize the methanol, and then methanol consumption begins at higher rate after the yeast is adapted to the medium change. DO suddenly undergo a sharp drop at this time that indicates a high consumption of methanol and increased fermentation speed.

As shown in Fig.6, the growth rate in the early hours has a negative value due to the yeast inadaptability. But the growth rate was increased quickly and then maintained at a constant level. Higher specific growth rate was observed in the fermentation process until 80th hour and its level was later decreased because of the increased concentration of the fermentation and higher level of toxic substances or oxygen deficiency. Fig. 7 shows an increase in dry weight of the cells compared with fermentation time. This increase reflects the successful growth of the cells. In the final fermentation hours, in addition to the lack of ascending growth in the diagram, a decrease is observed in the dry weight as the fermentation time passes, which indicates the end of the logarithmic growth phase and entering the stationary phase and eventually the completion of fermentation process. In Fig. 8, according to samples taken from the fermentor during the fermentation process, the number of cells was measured using turbidimetry. The obtained data indicate an increase in the number of cells in the logarithmic phase of growth. Considering the direct relationship between the bacteria growth and OD, the OD level was increased and with beginning of the stationary or death stage, the OD graph undergoes reduction and recession in line with the bacteria growth. Fig. 9 shows a comparison of the two parameters of OD and ww after in terms of fermentation time and we see that these parameters have a direct relationship with each other.

4 Conclusion

In this research, considering the transfer of technology from the production sector (production and research complex of Pasteur Institute of Iran), to achieve the amount of biomass produced in elapsed specified time, numerous experiments were carried out and their results have been reported in Table 1. As is evident, much time has been spent in some of the experiments and amount of the produced biomass has been increased. As presented in Table 3, the average ratio of methanol to biomass amount is 1.14, i.e. 89% and 11% of methanol has been converted to biomass and energy, respectively. It should be noted that methanol purity grade is 79%. Fig. 10 shows the results obtained from the different consumption methanol rates

at the end of fermentation for different batches before optimization.

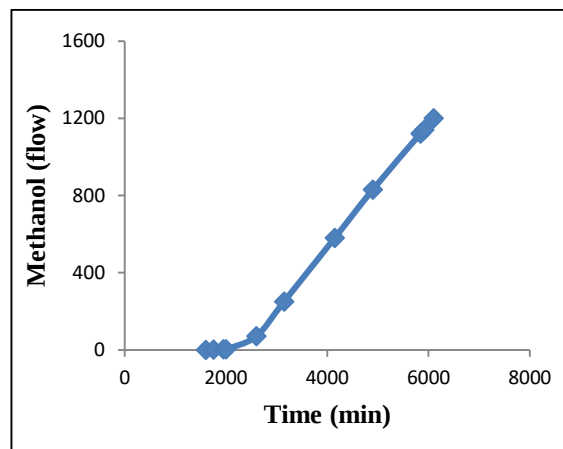


Figure 5: Methanol consumption rate during fermentation

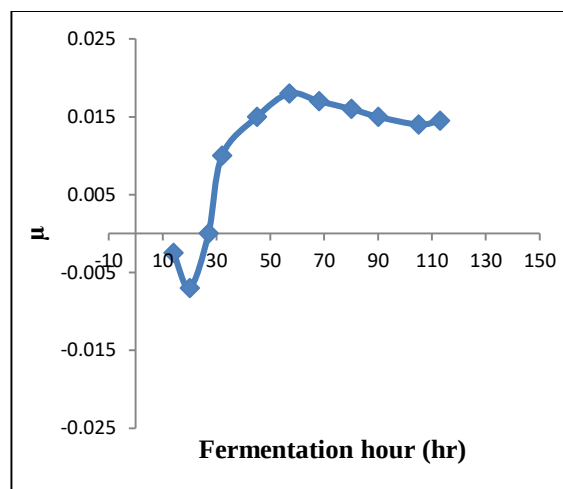


Figure 6: Trend of specific growth rate increase (μ)

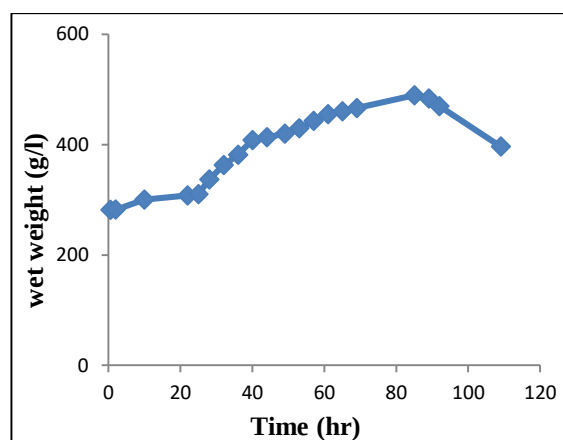


Figure 7: Relationship between the dry weight and fermentation time

Table 3: Methanol consumption rate and biomass specifications for different batches at the end of fermentation

Time (h)	w.w (g/l)	Biomass (kg)	Methanol consumption (L)	Methanol consumption/biomass (L/Kg)
131	292	1.54	2.03	1.13
132	270	1.50	2.15	1.04
135	349	1.74	2.28	1.18
139	280	1.57	2.32	1.04
140	350	1.77	2.40	1.24
144	296	1.67	2.5	1.07
147	290	1.66	2.61	1.17
153	310	1.81	2.73	1.96

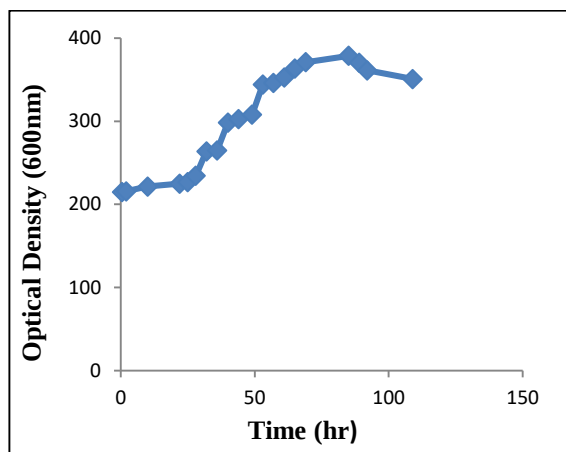


Figure 8: Relationship between OD and fermentation time

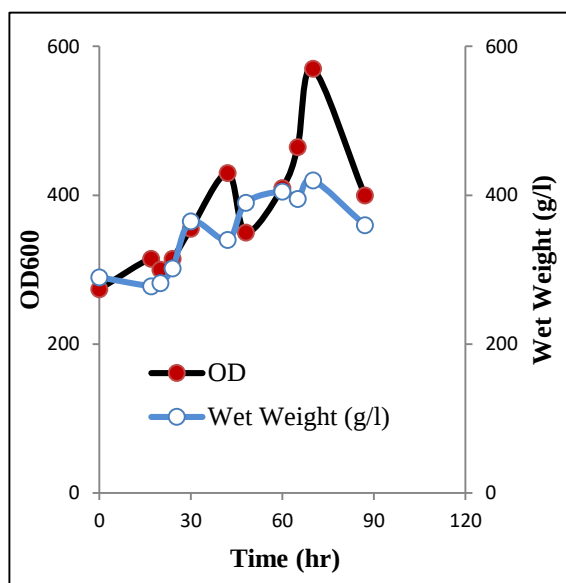


Figure 9: Comparison of dry weight with OD

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Ethical issue

Authors are aware of, and comply with, best practice in publication ethics specifically with regard to authorship

(avoidance of guest authorship), dual submission, manipulation of figures, competing interests and compliance with policies on research ethics. Authors adhere to publication requirements that submitted work is original and has not been published elsewhere in any language.

Competing interests

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Authors' contribution

All authors of this study have a complete contribution for data collection, data analyses and manuscript writing.

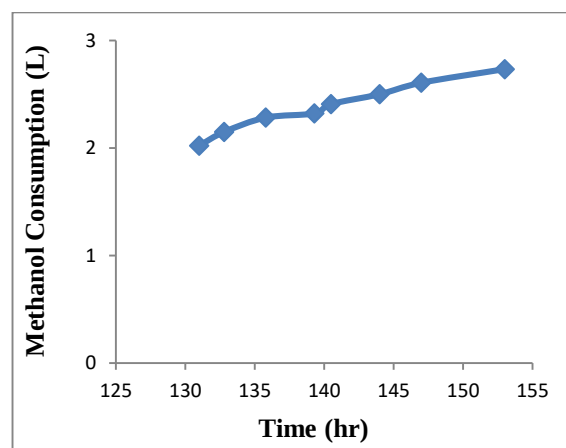


Figure 10: Pre-optimization methanol consumption rate diagram for different batches

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