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Bioprocess Development for High Cell Mass Production of the Probiotic Yeast-*Kluyveromyces lactis*

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Abstract : *In the present study, the key industrial strain of Kluyveromyces lactis NRRL Y-110 was used to develop an industrial process for probiotic cell mass production. Therefore, the biomass production process of K. lactis was developed via the optimization of different medium and process parameters in shake flask and bioreactor levels. In the first part of the work, the effect of medium composition on the production of biomass was investigated in shake flask culture. Among different production media studied, the medium composed of lactose, ammonium sulphate, magnesium sulphate, potassium dihydrogen phosphate and yeast extract yielded the highest volumetric high cell mass production of 4.34 g.L^{-1} after 24 hours cultivation. Furthermore, the optimization involved different key nutrients (carbon sources, nitrogen sources and mineral sources). The results obtained are helpful for the overproduction of highest cell mass by submerged culture of K. lactis on a semi-industrial scale. During bioreactor cultivation under controlled and uncontrolled pH, results showed that, high cell biomass yield of 15.1 g.L^{-1} was produced under controlled pH conditions compared to uncontrolled pH. This value was almost 48.30% higher than those obtained in controlled pH submerged shake flask culture.*

Keywords: *Kluyveromyces lactis, Probiotic, Biomass Production, Bioprocess optimization*

I. INTRODUCTION

Probiotics are viable microorganism or microbial mixtures which are able to colonize in the intestine and are administrated to improve the human body microbial balance by producing certain beneficial metabolites and to compete other pathogenic microorganisms. Therefore, they are considered as one of the main wellness products of growing interest in different countries [1]. For many years, most of research in this field was focused mainly on the isolation, biomass production and application of different bacterial probiotics especially those belonging to *Lactobacilli* such as *L. lactis* [2], *L. salivarius* [3], *L. delbrueckii* [4] and *L. plantarum* [5]. In addition to bacteria, different types of yeast strains were also proved to have probiotic/biotherapeutic activity when applied as single culture or in addition to other probiotic strains. These yeast strains are mainly: *Saccharomyces cerevisia, S. boulardii*, and *Kluyveromyces lactis*. Therefore, special interest was paid recently on research related to biomass production of probiotic yeasts in semi-industrial and industrial scales [6-8].

Kluyveromyces lactis is a yeast strain used in different industrial and research applications [9,10]. The name of this yeast was derived from its capability to assimilate lactose and its conversion into lactic acid. With the GRAS status given by the Food and Drug Administration (FDA), *K. lactis* has been known as a normal, even necessary component of many cultured dairy products" and yet "no reports of toxicity or pathogenicity have ever been associated with the presence of *K. lactis* has been reported in food [10,11]. Many studies had been done on the wide applications of *K. lactis* in industries. According to Van Ooyen *et al* [9], *K. lactis* acts as an excellent host for recombinant chymosin production in large scale, which was the first protein from higher eukaryote origin to be expressed in single cellular organisms. Merico *et al*. [12] have also reported that *K. lactis* is commercially used to produce lactase- and lactose-free milk and is also considered as a potential source for the production of α-galactosidase in food industry. In one report stated by Swinkels *et al*. [13], genetically modified *K.lactis* has been used to produce human serum albumin, human interleukin-1 beta, and hepatitis B surface antigen and other important biopharmaceuticals. Therefore, from an industrial point of view, the yeast biomass is a one fascinating raw material, having wide benefits and application which could be utilized in the production of foods, feeds, biochemical, etc. As being regarded as non-conventional yeast, scientific researches

on maximizing *K. lactis* biomass production have never been ceased. Recent publications showed pin-pointed nutrient requirements for *K. lactis* in biomass production. Therefore, a classical medium optimization study is needed for the determination of the best chemically-defined carbon, nitrogen, and vitamin sources for *K. lactis* cell growth. Experimentations on various carbon sources such as, galactose, glucose, lactose and sucrose have been conducted.

In many studies carried out on different carbon sources, culture medium with lactose and galactose has been experimentally proven to allow maximum biomass yield [14]. The effects of distinct nitrogen sources have been also tested by several authors in order to achieve high microbial mass production. Optimization of *K. lactis* culture medium encompasses both organic and inorganic nitrogen sources. Numerous studies have been performed to select the best nitrogen source which can maximize cell growth of *K. lactis*. These sources include ammonium chloride, ammonium nitrate, soybean meal, yeast extract and amino acids**.** Nowadays, studies are focusing much on the optimization of fermentation variables such as pH, temperature, agitation speed and fed-batch strategies. However, only a few contributed to high cell density after the optimization. On the other hand, the cost of materials has always been an issue. In this study, we will carry out a comparative study to maximize the *K. lactis* biomass production through a combination of medium optimization and fermentation parameter optimization. Several media with high potentials will be selected to undergo preliminary testing for their potential as high cell biomass promotors. This is followed by the optimization of the individual components of the best medium obtained. Finally, the cultivation of the probiotic strain *K. lactis* will be performed in 16-L bioreactor under controlled and un-controlled pH conditions.

II. MATERIALS AND METHODS

1. Microorganisms and Maintenance of Culture

Kluyveromyces lactis NRRL Y-110 was used throughout this study. This strain was obtained from the NRRL Culture Collection (ARS, Peoria, II, USA). This strain was maintained on yeast-malt agar (YMA) composed of ($g.L^{-1}$): glucose, 10; yeast extract, 3; malt extract, 3; peptone, 5 and agar, 20. The pH was adjusted manually by dropping 0.1 M of NaOH or HCl and was fixed to 7.0 before sterilization. The agar plates were inoculated with 0.1 mL of glycerine culture (working cell bank), spread and incubated at 30°C for 24 hours in order to obtain a good growth.

2. Preparation of Master and Working Cell Bank

The cell growth on the solid medium (YMA) was suspended with 50% glycerol and aliquots were stored at -80°C in 25 sterile vials (Cryogenic vials, Nalgene). This method of strain preservation has beneficial effects to avoid any changes in cell productivity and morphology. Pre-cultures were inoculated from these frozen stocks. These vials were utilized as inocula for all subsequent experiments. Master Cell Bank and Working cell culture preparation was carried out in Class II Biological Safety Cabinet using aseptic techniques at Wellness Industry Culture Collection, IBD, Johor, Malaysia.

3. Cultivation Media

3.1. Seed culture of *Kluyveromyces lactis*

The medium composed of the ($g.L^{-1}$): lactose, 30; yeast extract, 1; dipotassium phosphate, 2; ammonium phosphate, 1; diammonium phosphate, 1 and magnesium sulphate heptahydrate, 0.1. The seed cultures of *K. lactis* were transferred to the seed medium by pipetting about 5 mL of the seed culture with a sterile tip. The seed culture was grown in a 250 mL of Erlenmeyer shake flask with a working volume of 50 mL of the liquid medium. The flask was incubated at 30°C on a rotary shaker (New Brunswick), agitated at 200 rpm for 24 hours. This procedure was used for the preparation of standard inocula for all experiments along this fermentation process.

3.2. Media for biomass production

For primary screening, five broth media from previous work concerning the cultivation of *K. lactis* strains have been chosen for further optimization. All these media have been previously reported for their high support for biomass production of *K. lactis* species. The main components of these media were tabulated in Table 1. The growth of *K. lactis* was carried out in duplicates of each media. According to standard procedure, the carbon sources either lactose or glucose has been autoclaved separately to prevent mailard reaction. After cooling to about 40°C, the carbon source was aseptically added to the cultivation medium directly before inoculation. The initial pH of all media used was adjusted to 7.0 before sterilization ex-situ using autoclave (Hirayama, HVE-50).

Tab. 1**:** Different cultivation media used for screening experiment

Media	Compositions $g.L^{-1}$	Reference
Medium 1	Yeast extract, 10; peptone, 10; cane molasses, 100; corn steep liquor, 100.	[15]
Medium 2	Lactose, 37.1; yeast extract, 5; peptone, 5; dipotassium phosphate, 5; ammonium phosphate, 5.	[16]
Medium 3	Glucose, 100; sodium phytate, 12; yeast extract, 10; diammonium suphate, 15; ammonium chloride, 11; magnesium sulphate heptahydrate, 1.3; potassium chloride, 2.5; calcium chloride, 2.5.	[17]
Medium 4	Lactose, 28.2; potassium dihydrohen phosphate, 5; ammonium phosphate, 8.8, magnesium sulphate heptahydrate, 0.4; yeast extract, 17.	[18]
Medium 5	Lactose, 60; ammonium sulphate, 4; magnesium sulphate heptahydrate, 2; potassium dihydrogen phosphate, 4; yeast extract, 2.	[19]

4. Medium Optimization

The optimization process was carried out for medium number 5, which yielded the highest cell mass [19] in shake flask culture at 30°C in a shaking incubator with a rotational speed of 200 rpm for 24 hours (Table 1). Effects of different concentration of carbon sources on liquid culture of *K. lactis* were studied using 0-100 $g.L^{-1}$ of lactose. The other culture medium components were ($g.L^{-1}$): ammonium sulphate, 4; $MgSO_4$, 2; K_2HPO_4, 2; yeast extract, 4. Concerning the effect of different yeast extract concentrations, yeast extract was used at levels of 0, 1, 2, 3, 4, 5, 6, 7, 8 and 9 $g.L^{-1}$. The concentrations of $(NH_4)_2SO_4$, $MgSO_4.7H_2O$ and KH_2PO_4 were varied ($g.L^{-1}$) from 0-8, 0-4 and 0-6, respectively. Inoculation was performed by transferring 5 mL of seed culture to 45 mL medium in a 250 mL Erlenmeyer flask.

5. Growth Kinetics in Submerged Shake Flask Cultivation

The growth kinetics of *Kleuyveromyces lactis* were studied in shake flask cultures using non-optimized and optimized medium. Cultivations were carried out at 30°C, 200 rpm for 24 hours.

6. Bioreactor Cultivation and Process Conditions

In situ sterilizable stainless steel stirred tank-bioreactor 16-L (BioEngineering, Wald, Switzerland) was used in this study. Before inoculations, the bioreactor was filled with the optimized medium and sterilized at 121°C for 15 minutes. The bioreactor was equipped with pH and dissolved oxygen probe (Ingold, Mettler-Toledo, Switzerland), foam sensor, and stirrer with 6 bladed Rushton turbines impellers. Sterile air was spurge at approximately 1.0 ($vv^{-1}min^{-1}$), the agitation speed was set at 200 rpm and the temperature was adjusted to 30°C. Foam was suppressed using silicon base antifoam agent (Silicone A, Sigma, USA). *K. lactis* was cultivated under uncontrolled and controlled pH conditions. In case of pH controlled culture, the pH was adjusted to 5.5 by continuous addition of 2 M NaOH and 2 M HCl using peristaltic pumps. The initial dissolved oxygen for both cultures was set at 100% at the beginning of cultivation.

7. Analytical Procedure

7.1. Cell dry weight determination

For submerged culture in both shake flasks and bioreactors, samples in the forms of 2 flasks, or 50 mL in the case of bioreactor, were taken at different time intervals. Cell growth was monitored by measuring the optical density (OD) of the cell suspensions in a single beam spectrophotometer (DR 6000, Hach Co., Loveland, CO, USA) at 600 nm. A series of dilutions has been done at ratio 1:10 and 1:100 for better accuracy. The OD of the culture was converted to dry cell mass through a previously prepared linear correlation between OD and CDW. One OD_{600} nm was almost equal to 0.3 $g.L^{-1}$ for this culture.

7.2. Glucose and lactate determination

Both glucose and lactic acid were determined by HPLC. In case of lactic acid, a 250 mm × 4.6 mm ID spherisob Octyl Column (Waters, Milford, MA, USA) and a UV detector (210 nm) were used. The adsorbed substances were eluted with 0.2 M H_3PO_4 at flow rate of 0.8 $mL.min^{-1}$ at room temperature. For glucose, a 400 mm × 4.0 mm ID μ Bondapak/Carbohydrate column (Waters, Milford, MA, USA) with IR detector were used. The mobile phase used was acetonitrile : water (80:20) at a flow rate 1.0 $mL.min^{-1}$ at room temperature.

III. RESULTS AND DISCUSSION

1. Cultivations in Different Media

According to Huang and Tang [20], cultivation media should contain all the necessary nutrients to maintain cell growth and to support product biosynthesis. There are two types of growth media that can be classified into: synthetic (with a well-defined composition) and complex media (media include some components of not completely defined compositions). Defined media are described as to have specific amounts of pure chemical compounds and an identifiable chemical composition. Complex (enriched) media contain

natural compounds which are rich in different nutrients but their fully chemical components are not determined and sometimes are source and suppliers dependent [20]. Generally, defined media are used to obtain high cell density because the nutrient concentrations are known and can be controlled during cultivation [21]. As for complex media, such as peptone and yeast extract can vary in composition and quality making fermentation less reproducible [22].

In the present work, comparison between synthetic medium and rich complex medium has been studied. Subsequently, medium 1 contained rich complex nutrient while medium 5 contained synthetic components. Compositions of various media gave results contradicting with the comparative study carried out by Rollini *et al.* [23]. Although Rollini *et al*. [23] reported that the application of cheese whey as a carbon source will result in about 8- to10-fold increase in cell growth of *K. lactis* than lactose, our experimental results (Fig. 1) showed the opposite. This may be attributed to the fact that the variation in the quality of rich complex media is the main factor as vague concentration of nutrients in rich complex medium providing different nutrients to yeast cells.

Based on the experiments of previous reports, five production media (Table 1) were selected and tested for their potential to support *K. lactis* growth. Figure 1 showed that all production media supported the growth of *K. lactis* at different extents. This experiment solely serves to determine the best production media among the five for further optimization. The comparison between different high cell mass production, lactate yield, lactose utilization and the final pH after 24 hours of fermentation at 30°C in shake flasks (250 mL) used in this study is represented in Figure 1. The results show the effect of different carbon sources on *K. lactis* biomass yield. In this experiment, Media 2, 4 and 5 contained lactose as carbon source while media 3 contained glucose as carbon source. Figure 1 showed that there were significant differences between glucose and lactose as carbon sources in terms of biomass yield. These results are in accordance with those reported by Spencer *et al*. [24], who found that *K. lactis* metabolized lactose preferably as well as glucose. As shown in Figure 1 the highest production of biomass of about 4.34 $g.L^{-1}$ was obtained in medium 5, containing lactose, ammonium sulphate, $MgSO_4$, K_2HPO_4, and yeast extract, followed by medium 1 which yielded about 4.0 $g.L^{-1}$. Based on the highest production of cell mass, medium 5 was chosen for further optimization studies.

2. Kinetics of Cell Growth and Lactate Production in Shake Flask Culture in Non-Optimized Medium

Experimental results of *K. lactis* cell growth obtained from different literature varied depending on different carbon and nitrogen sources applied. Moreover, few reports investigating the optimal nutrient concentrations by trial and error methods have been found in the literature. This experiment was devoted to investigate the kinetics of cell growth and lactate production for *K. lactis* cultivated on medium 5. Figure 2 shows the cell dry weight, lactate yield and pH changes in shake flask fermentation for 48 hours. Sampling was taken every 6 hours to observe the cell growth pattern. As can be observed, biomass yield increases significantly after 6 h and doubles at 12 h. Subsequently, the biomass yield reaches its optimal level 4.70 $g.L^{-1}$ at 36 h, and then eventually declines to 4.29 $g.L^{-1}$ during the following cultivation period. This indicates that 36 h is the cell growth peak for *K. lactis,* producing maximal biomass yield. The relationship between lactate yield and medium pH is inversely proportional. As biomass yield increases from 0 to 36 h, lactate yield follows the positive trend while medium pH decreases significantly. Spencer *et al*. [24] reported that when *K. lactis* gains energy by metabolizing lactose, it will grow forming cell biomass, with the production of lactate as metabolic residue.

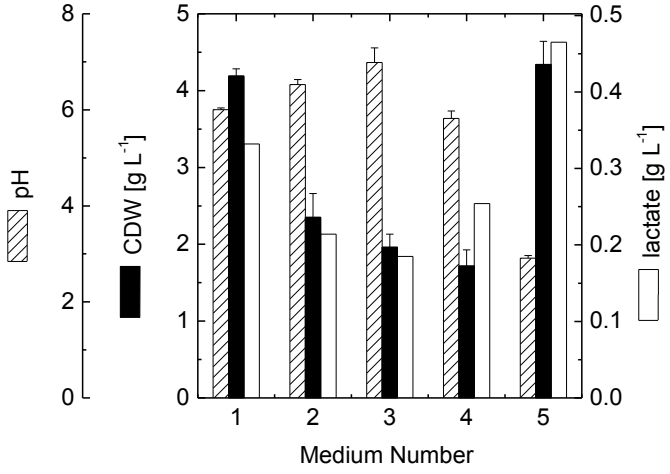

Fig. 1: Cell dry weight (CDW), lactate yield, and final pH in 5 different media after 24 hours of fermentation at 30°C in Erlenmeyer shake flask.

3. Effect of Different Lactose Concentrations on Biomass Production

Medium optimization was carried out using the classical approach, by changing a single medium component at a time. The purpose of the experiment is to determine the optimal concentration of lactose in medium 5 required to maximize biomass yield of *K. lactis*. Figure 3 illustrates the cell dry weight obtained at different concentrations of lactose ranging from 0 to 100 $g.L^{-1}$ after 48 hours of incubation at 30°C. The inocula (10%) of *K. lactis* were cultivated in media containing different lactose concentrations, and the flasks were then incubated. The utilization of lactose as carbon source is a characteristic trait for *K. lactis* and has been studied extensively [25]. Various authors investigated the effect of changing the initial lactose concentration on *K. lactis* biomass production. At 10 $g.L^{-1}$ initial lactose concentration, the cell dry weight of 2.98 $g.L^{-1}$ was obtained. However, increasing the lactose concentration resulted significantly increased the cell biomass, reaching its maximum (5 $g.L^{-1}$) at 60 $g.L^{-1}$ initial lactose concentration. On the other hand, increasing lactose concentration above 60 $g.L^{-1}$ did not have any noticeable effect on biomass production. These results were in accordance with those reported by Lukendoh *et al*. [26]. Moreover, Rech and Ayub [27] also observed similar growth patterns upon increasing the lactose concentration. The results that cell growth biomass did not increase significantly after increasing lactose above 60 $g.L^{-1}$ can be attributed to the fact that higher lactose concentrations used in batch cultured yeasts can result in Crabtree repression which in turn inhibits the respiratory enzymes [26]. Additionally, higher lactose concentrations can lead to the osmotic inhibition of cell growth [28]. From these results, 60 $g.L^{-1}$ initial lactose concentration was chosen as the optimum concentration for further cultivations.

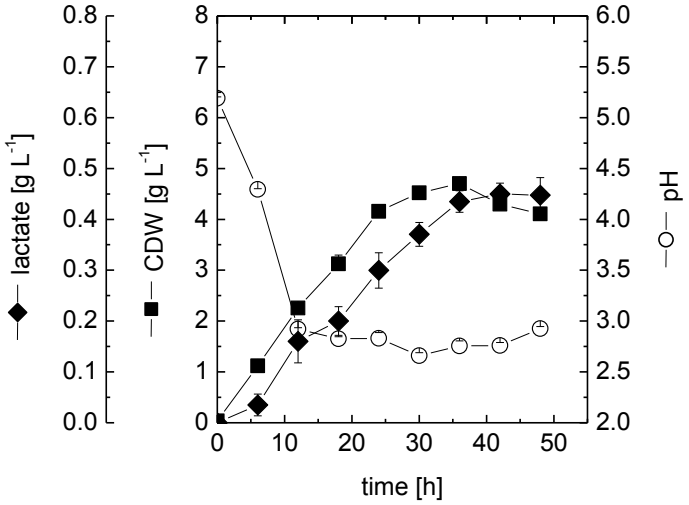

Fig.2: Cell dry weights (CDW), lactate yield, pH changes in non-optimized medium every 6 hours during fermentation in Erlenmeyer shake flask at 30°C for 48 hours

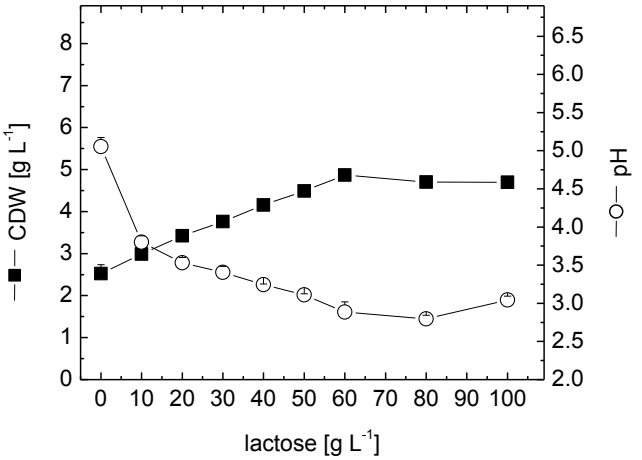

Fig. 3: Cell dry weight and final pH at different concentrations of lactose after 48 hours of fermentation at 30°C in Erlenmeyer shake flasks.

4. Effect of Different Yeast Extract Concentration on Growth of *K. lactis*

Nitrogen requirements have come under special scrutiny because of their effects on cell growth. In the following experiment, organic nitrogen sources and inorganic nitrogen sources were tested, i.e. yeast extract and ammonium sulphate, respectively. Yeast extract is the most commonly used nitrogen source in fermentation as it provides growth factors that are suitable for microbial growth [29]. Nevertheless, there are uncertainties estimating the optimal concentration of yeast extract required to allow maximal cell growth. Rosma and Cheong [30] suggested that the initial yeast extract concentration ranges from 0 to 10 $g.L^{-1}$. The purpose of this study is to investigate the effect of different concentrations of yeast extract on cell biomass of *K. lactis*. Figure 4 illustrates the cell dry weight obtained at different concentrations of yeast extract ranging from 0 to 10 $g.L^{-1}$ after 48 hours incubation at 30°C. Increasing yeast extract concentration resulted in a significant increase in the produced cell biomass. The cell dry weight increased from 1.68 $g.L^{-1}$ obtained at 0.0 $g.L^{-1}$ initial yeast extract and reached its maximum (5.2 $g.L^{-1}$) upon using 4.0 $g.L^{-1}$ initial yeast extract concentration. Increasing yeast extract concentration above 4.0 $g.L^{-1}$ gradually decreased the concentration of the obtained cell biomass. Therefore, further experiments were performed at an initial yeast extract concentration of 4 $g.L^{-1}$.

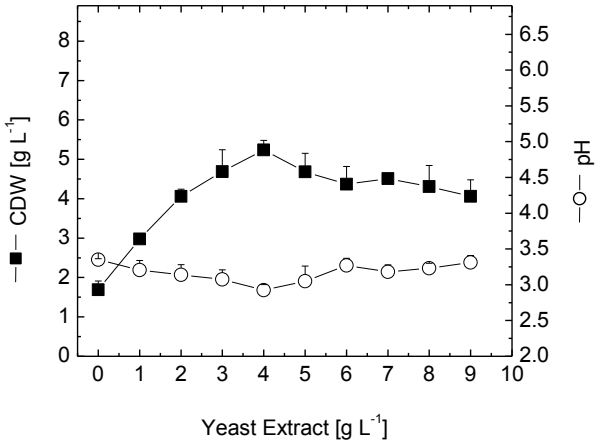

Fig. 4: Cell dry weight and final pH at different concentrations of yeast extract after 48 hours of fermentation at 30°C in Erlenmeyer shake flasks.

5. Determination of Optimal Ammonium Sulphate Concentration for High Cell Mass Production

Ammonium sulphate is one of the most important inortanic nitrogen sources used, and it has been found to permit maximal biomass production. The effect of inorganic nitrogen sources on *B. thuringiensis* has been previously reported [31]. The authors suggested that the effect of the ratio of organic to inorganic nitrogen sources is far more important than the ratio of carbon to nitrogen sources (C/N). However, little or no information has been reported about the effects of these interactions on *K. lactis* biomass production. Figure 5 illustrates cell dry weight and final pH obtained at different initial concentrations of ammonium sulphate ranging from 0 to 8 $g.L^{-1}$. The cultivation was conducted for 24 h. Increasing the concentration of ammonium sulphate gradually increased the concentration of the produced cell biomass. The climax of cell biomass of 5.61 $g.L^{-1}$ was obtained upon using 4 $g.L^{-1}$ ammonium sulphate. Concomitantly, the pH value of the culture reached its minimum (2.74) at the same initial concentration. Further increase in the ammonium sulphate initial concentration resulted in a significant decrease in cell biomass production, reaching its minimum (2.8 $g.L^{-1}$) at 8 $g.L^{-1}$ ammonium sulphate. Again the pH reacted inversely to the growth pattern and increased at 8 $g.L^{-1}$ ammonium sulphate reaching 3.75. Accordingly, 4 $g.L^{-1}$ has been chosen as the best concentration for subsequent experiment.

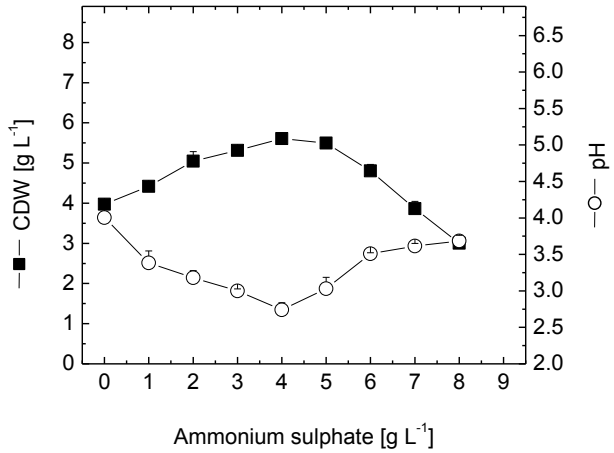

Fig. 5: Cell dry weight and final pH at different concentrations of ammonium sulphate after 48 hours of fermentation at 30°C in Erlenmeyer shake flasks.

6. Effect of Different Magnesium Sulphate Concentrations on Cell Growth

Magnesium sulphate as the micronutrient supplemented to *K. lactis* has been stressed by several authors. In *K. lactis* physiological studies, micronutrient supplementation has proven to enhance the cell division, thus enhancing cell growth. However, in recent reports concerning the development of high cell density cultivation (HCDC), the influence of magnesium sulphate on yeast cell growth has been rarely discussed. This experiment aims to study the effect of changing magnesium sulphate concentrations on *K. lactis* cell growth. Figure 6 illustrates the cell dry weight and final pH obtained at different magnesium sulphate initial concentrations, ranging from 0 to 4 $g.L^{-1}$ after 48 hours of incubation at 30°C. The concentration of cell biomass increased markedly upon increasing the initial concentration of magnesium sulphate from 0 to 1.5 $g.L^{-1}$, where the maximal cell biomass production of 5.77 $g.L^{-1}$ was obtained. Further increase in the concentration of $MgSO_4$ resulted in slight decrease in the maximal cell growth obtained, which remained more or less constant with higher $MgSO_4$ concentrations. Thus, 1.5 $g.L^{-1}$ $MgSO_4$ has been chosen as the best concentration.

7. Effect of Different Potassium Dihydrogen Phosphate Concentrations on Cell Growth

Many reports have investigated the effect of the addition of inorganic phosphate sources on microbial growth and concluded that inorganic phosphate stimulates cell assimilation, thus leading to higher cell population. Manera *et al*. [18], reported that the addition of potassium dihydrogen phosphate increased cell assimilation of *K. marxianus*, which results in higher cell biomass production.

Nonetheless, there are ambiguities regarding optimal concentration of potassium dihydrogen phosphate, which should be added into the medium to enhance the yield. Additionally, few reports investigated the effect of phosphate addition on the growth and biomass production of *K. lactis*. Figure 7 illustrates the effect of different initial concentrations of potassium dihydrogen phosphate on cell biomass production of *K. lactis.* Different cultivation media containing different initial concentrations ranging from 0 to 6 $g.L^{-1}$ have been carried out for 48 hours at 30°C. The results obtained showed that increasing initial potassium dihydrogen phosphate concentration from 0 to 2 $g.L^{-1}$ significantly increased cell biomass production from 4.95 to 6.84 $g.L^{-1}$, respectively. Thereafter, further increase in potassium dihydrogen phosphate initial concentration gradually decreased cell biomass production. The pH value reacts inversely to the cell growth pattern. Accordingly, initial concentration 2 $g.L^{-1}$ has been chosen as the optimal potassium dihydrogen phosphate concentration.

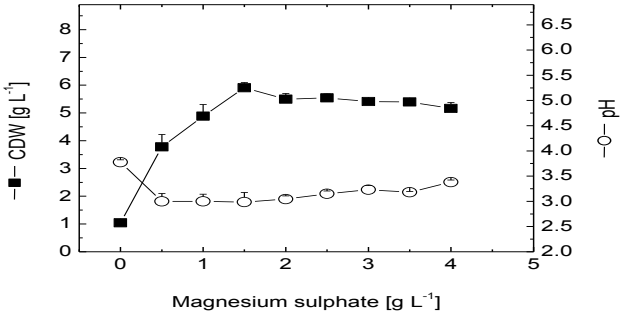

Fig. 6: Cell dry weight and final pH at different concentrations of magnesium sulphate heptahydrate after 48 hours of fermentation at 30°C in Erlenmeyer shake flasks.

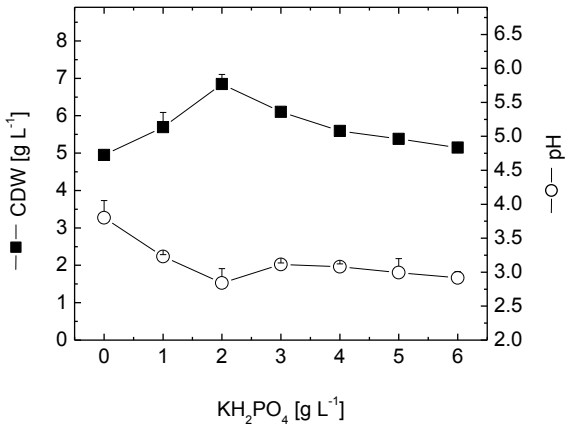

Fig. 7: Cell dry weight and final pH at different concentrations of Potassium dihydrogen phosphate after 48 hours of fermentation at 30°C in Erlenmeyer shake flasks.

8. Comparison between Cell Growth Kinetics in Non-Optimized and Optimized Media

The present experiment aims to compare between the cell growth kinetics of *K. lactis* cultivated under non-optimized and optimized media. Figure 8 illustrates the cell dry weight, lactate yield as well as pH changes in optimized medium during 48 hours incubation time. The finally optimized medium consists of ($g.L^{-1}$): lactose, 60; yeast extract, 4; ammonium sulphate, 4; magnesium sulphate heptahydrate, 1.5; potassium dihydrogen sulphate, 2. As can be observed, the maximal cell dry weight (6.32 $g.L^{-1}$) was recorded after 35 h of cultivation. This represents an increase in cell biomass production of about 34.45% from the cell biomass previously obtained under non-optimized medium (4.70 $g.L^{-1}$ at 36 h, Fig. 2). Applying the optimal concentrations of different medium components provides suitable amounts of the nutrients with proper metabolic balances to *K. lactis* cells, which will result in the maximal production of cell biomass, as well as decreasing the probability of osmotic inhibition. Additionally, the pH parameter changes according to the production of lactate. However, the slight decrease in the pH value from those obtained in non–optimized medium was insignificant as the deviation percentage is less than 1%.

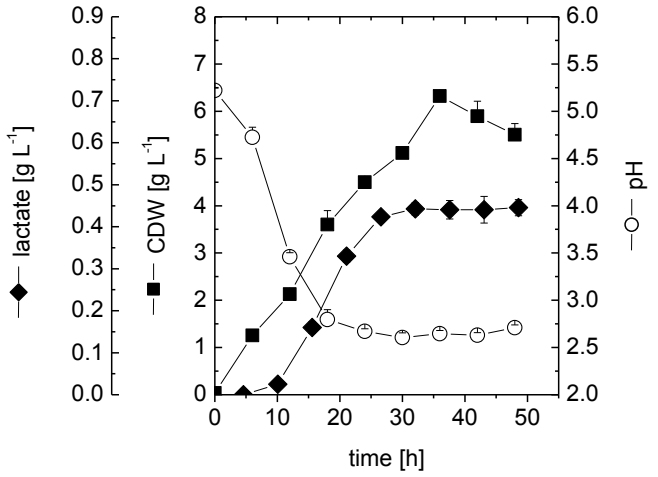

Fig. 8: Cell dry weight (CDW), lactate yield, pH changes during cultivation of *K. lactis* in Erlenmeyer shake flask using optimized cultivation medium.

Tab. 2: Maximum Cell Dry Weight (CDW), Specific Growth Rate (μ_{net}) Biomass yield ($Y_{X/S}$), Doubling time (t_d), Final pH in medium before and after optimization.

Parameters	Non-optimized	Optimized
Maximum CDW (gL^{-1})	4.70	6.33
Specific Growth Rate, μ_{net} (h^{-1})	0.14	0.16
$Y_{X/S}$	0.07	0.10
Doubling time (h), t_d	4.89	4.1
Final pH	2.92	2.71

Table 2 represents the comparison of different growth kinetic parameters obtained for *K. lactis* grown on non-optimized and optimized media. The optimization of different medium components significantly increased the specific growth rate and biomass yield by about 19.20% and 34.66%, respectively. Moreover, cellular doubling time decreased by about 16.20% (from 4.89 to 4.1 h), indicating that the yeast cells are present in a better physiological state that leads to faster reproduction.

9. Comparison between the Effects of Controlled & Uncontrolled pH during Fermentation in a batch Cultivation using 16-L Stirred Tank Bioreactor

This experiment was designed to investigate the effect of controlling the pH of the cultivation in comparison with the uncontrolled conditions. The cultivations were run in 16-L stirred tank bioreactor. The controlled pH experiment was adjusted to 5.5, as previously described in Materials and Methods section. Figure 9 illustrates biomass yield, lactate yield and pH changes in 16 L bioreactor under uncontrolled pH at 30°C for 48 hours. Figure 10 represents biomass yield, lactate yield and pH changes under controlled pH conditions at 30°C for 48 hours. In both experiments, samples were taken for every 4 hours. The cell biomass yield obtained from both bioreactor fermentations was much higher than the results obtained from shake flask cultivation under optimized medium. The maximal cell biomass obtained from bioreactor cultivations recorded 15.14 and 10.21 $g.L^{-1}$ for pH controlled and uncontrolled fermentations, respectively. This biomass yield represents an increase by about 139.6 and 61.49% from the maximal biomass yield obtained in shake flask cultivation (6.32 $g.L^{-1}$, Fig. 8). This can be attributed to the better cultivation conditions present in bioreactor in terms of better oxygenation, agitation, and mixing and distribution of the medium components as well as metabolized products. Additionally, the significant increase in cell biomass production in the pH-controlled fermentation can be explained based on the fact that lactate production creates a highly acidic environment which inhibits the cell growth. Hensing *et al.* [32] reported that β-galactosidase enzyme was inactivated under pH values lower than 3. The pH controlled medium is found conducive for optimal cell biomass production and of β-galactosidase activity.

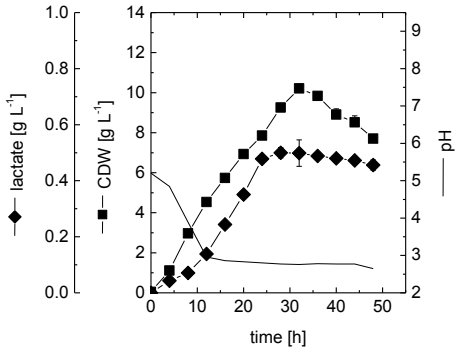

Fig. 9: Cell dry weight (CDW), lactate yield, pH changes in optimized medium every 4 hours during fermentation in a bioreactor at 30°C for 48 hours under uncontrolled pH.

As observed, batch cultivation has been contrasted under two conditions in terms of growth kinetics: uncontrolled and controlled pH conditions. Under controlled pH conditions, relative incremental 55.38% (specific growth rate) and 48.82% (biomass yield) have been observed, indicating relatively high cell growth rate. On top of that, yeast cells double at a rate 35.67 % faster than uncontrolled pH conditions. To summarize, *K. lactis* in batch mode is better cultivated under controlled pH condition

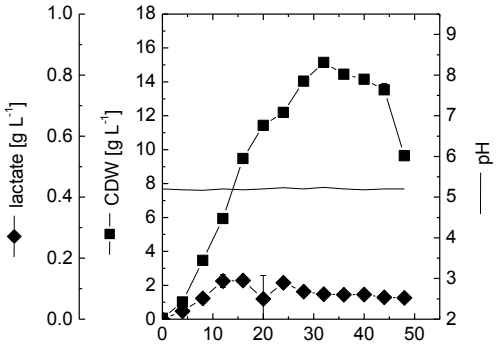

Fig. 10: Cell dry weight (CDW), lactate yield, pH changes in optimized medium every 4 hours during fermentation in a bioreactor at 30°C for 48 hours under controlled pH.

IV. CONCLUSION

In this research, a process for submerged cultivation of yeast probiotic, *K. lactis* for the production of high cell mass was demonstrated. The effects of medium components, carbon source, nitrogen source and minerals, on supported high cell mass were investigated in order to obtain a new optimized production medium. As a conclusion, maximum biomass yield can be achieved by *Kluyveromyces lactis* cultivation using cultivation conditions: lactose, 60 $g.L^{-1}$; yeast extract, 4 $g.L^{-1}$; ammonium sulphate, 4 $g.L^{-1}$; magnesium sulphate heptahydrate, 1.5 $g.L^{-1}$; potassium dihydrogen phosphate, 2 $g.L^{-1}$; at 30°C under controlled pH at 5.2. The fundamental results obtained in this research are beneficial for further development of probiotic yeast cultivation strategy for the overproduction of high cell mass probiotic cell on a pilot scale.

Tab. 3: Overview of growth kinetics parameters in both shake flask and bioreactor scale.

Parameters	Shake Flask		Bioreactor (16 L)	
	Non Optimized	Optimized	Uncontrolled pH	Controlled pH
X_{max} ($g.L^{-1}$)	4.70	6.33	10.21	15.14
Specific growth rate, μ_{max} (h^{-1})	0.14	0.16	0.32	0.50
$Y_{X/S}$ ($g.g^{-1}$)	0.07	0.10	0.17	0.25
Doubling time (h), t_d	4.89	4.1	2.13	1.37
Final pH	2.92	2.71	2.65	5.5

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