Production of Ethanol from Sugars Fermentation By Yeasts Using Bioreactor

استخدام الخمائر في انتاج الايثانول من تخمر السكر وباستعمال المفاعل الحيوي

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Abstract

Two yeast isolates *of C.kefyr*-T and *C.kefyr*-S, were isolated from crude whey, the optimum pH value was 5, and *S.cerevisiae* was significantly best in ethanol and Biomass production from all glucose concentrations compared to *C.kefyr*-T and *C.kefyr*-S which were significantly best ethanol and biomass production from all galactose concentrations comparing with *S.cerevisiae*. *C.kefyr*-T which was best in production compared to *C.kefyr*-S. Best ethanol production was 8.7% Ethanol from 10% sugar mixture, by mixed culture of (*S. cerevisiae*+ *C.Kefyr*-T), and best ethanol production was 4.6% from 8% synthetic lactose by mixed culture of (*C.kefyr*-T+*C.kefyr*-S). There is a significant decrease in ethanol and biomass production from all mixtures by using mixed culture of (*C.kefyr*-T+ *C.kefyr*-S+ *S. cerevisiae*) than using mixed culture of (*C. kefyr* + *S. cerevisiae*) and mixed culture of (*C.kefyr*-T + *C.kefyr*-S). Different period of times at (24, 36, 48, and 60) h of fermentation in bioreactor are presented. The initial sugar mixture concentration was 10% in all cases. The ethanol production during the 48 h gave a maximum value of 15.5 % by mixed culture of (*C. kefyr* + *S. cerevisiae*), but it remains at low levels within other periods, whereas the highest biomass 16% gained at the same bioreactor conditions.

المستخلص

تم استخدام نوعين من الخمائر المستخلصة من بقايا الحليب في انتاج الايثانول عند الدالة الحامضية المثلى 5 في المفاعل الحيوي . افضل انتاج للايثانول كان 8.7% من 10% من الكلوكوز وذلك بمزج (*S. cerevisiae*+ *C.Kefyr*-T) اما افضل انتاج للايثانول كان 4.6% من 8% كالكتوز باستعمال المزيج (*C.kefyr*-T+*C.kefyr*-S). لوحظ نقصان ملحوظ في الايثانول وانتاج الكتلة الحيوية من الخليط الكلي باستخدام المزيج (*C.kefyr*-T+ *C.kefyr*-S+ *S. cerevisiae*). تم دراسة اوقت المثل لعملية التخمير(24،36، 48، و60) ساعة . لوحظ ان الوقت الافضل لعمليات التخمير هي 48 ساعة حيث اعطى اعلى قيمة انتاج 15.5% وذلك بمزج (*C. kefyr* + *S. cerevisiae*).

Introduction

Alternative fuels are important for the world to reduce their dependence on fossil fuels. Currently, ethanol is the only renewable fuel that is produced in commercial quantity. The demand for ethanol is increased throughout the world. The production of ethanol is limited by the available feedstocks and processing technology. Ethanol is a substitute of gasoline where the world consumption of ethanol as motor fuel is (30) tons, and the production of ethanol increased by 11% in the past five years [1]. The United States Environmental Protection Agency Act 2005 mandated a production of 4.0 billion gallons of ethanol in 2006 increasing to 7.5 billion gallons in 2012 [2]. On the other hand ethanol is one of most important organic chemicals, and has a wide spread applications in (food, pharmaceutical and chemical) industries, use as solvent of substances included in (perfumes, drugs, and make up) industry, and as feedstock for the synthesis of other organic products as (acetic acid) [3]. More than half of industrial ethanol is produced through fermentation of any organic material with high carbohydrates content, yeast in used in this fermentation for their ability to ferment wide range of sugar [4]. In an aerobic condition, they convert sugar to ethanol, carbon dioxide, and glycerol, while convert to biomass and carbon dioxide under aerobic conditions [5]. The industrial importance of yeast are: their ability to ferment wide range of sugar for, ethanol, single cell protein (feed source for human and animal), enzymes, fats, and vitamins production [6]. The yeast has the ability to convert lactose directly to ethanol is limited [7]. There are several yeast strains with ability of lactose fermentation [7] are: *Kluyveromyces Lactis*, *Kluyveromces fragilis*, *Kluyveromces Marxianus*, *Candida kefyr*, *Candida pseudotropicalis* .Most studies focused on using species such as : *C. peudotropicalis*, *K. Marxianus*, and *K. fragilis*. And few interested studies with *C.kefyr*.*C.kefyr* belong to *Candida* Genus which belongs to *Cryptococcaceae* Family, and imperfect fungi class, which are asexual reproduction. *C.kefyr* present as microflora in cow, sheep and goat milk [8],*C.kefyr* influence the flavor profile of the fermented milk, through ethanol and carbon dioxide production[9].

Materials & Methods

Saccharomyces cerevisiae of trade mark name yuva (Turkey), and two isolated lactose fermenting yeasts were isolated from whey sample according to [10]and were identified in (Central Health Lab./ Identification Unit- Fungi) to (*Candida Kefyr*-T) and (*Candida kefyr*-S). Direct counting method, i.e. spread plate technique was used to determine the number of viable cells, in which a few serial dilutions were made before spreading. After the preset incubation time at 30 °C, colonies grown in Petri dishes were used to count the number of viable cells and expressed as colony forming units (CFU). 5% of three individual yeast inoculums were prepared, calculated number of cells (2*107) cell/ml (by Thoma's chamber as At least three measurements were made for each condition and the data given were an average values [11].

Different concentrations (2,4,6,8,10,12,14)% of glucose were prepared, by disolving different grams of this sugar in (100)ml of synthetic medium in (1000) ml of D.W according to[12]. They Justified at different (3, 4,5,6,7) pH values and sterilized by autoclave under 15 psig at 121°C for 15 minutes. Then each conical with different sugar concentrations were inoculated by 5 ml of one type (*S.cerevisae*, *C.kefyr*-T, *C.kefyr*-S) inoculums, and Incubated at 30°C for (48)hr. The same experiments were repeated by using galactose instead of glucose. Different concentration (2,4,6,8,10,12,14) % of equal ratio (glucose & galactose) mixture were prepared, justified at pH=5, each seven different concentrations were inoculated by one type of yeast inoculums, then incubated as above. Mixed sugar were prepared and pH values were justified as above, each concentration was inoculated by mix

(*S.cerevisiae* + *C. kefyr*-T) or (*S.cerevisiae* + *C.kefyr*-S) or (*C.kefyr*-T + *C.kefyr*-S) or (*S. cerevisiae*+ *C.kefyr*-T+*C. kefyr*- S), then incubated as the same condition above.

Ethanol production by bioreactor process

For the fermentation (ethanol production) stage, 5L thermoregulated double-jacket cylindrical agitated bioreactors (Innobio, Biotechnology Innovation, Korea) with 2 working volume were used. The temperature and agitation speed were 35°C and 180 rpm, respectively. Period of fermentation was tested at different times of (24, 36, 48, 60) h. The sterilization of bioreactor and in which medium was performed in an autoclave (LabTech , Daihan Labtechc Co. , Korea) at 121°C for 15 min. pH was adjusted to 5.0 before the addition of 100 mL of yeast culture grown aerobically under submerged optimized conditions. The temperature and pH were kept stable during fermentation [13]. Inoculums were added aseptically using a sterile method as recommended by Manufacturer Company. The experiments with sugar as the carbon source in the bioreactor stage were carried out with (glucose + galactose) mixture in ratio (1: 1). Initial concentrations of total sugar was (10 % w/v), and mixed culture of (*C. kefyr*-T + *S. cerevisiae*) were studied for the optimization of the bioreactor process.

Ethanol concentration was quantitly measured by method of analysis of American Society of Brewing chemists in yeast culture dependence on specific gravity measurement by using pychnomenter [14]. After centrifuged of 10 ml fermented sample at 3500 rpm for 15 minutes, then separated supernatant to calculate ethanol concentration. Ethanol concentration was qualitatively measured by using gas chromatography at (Ministry of Sciences & Technology), the results were closed to those which obtained by using Pychnometer, as shown in table (1).

Table (1): comparing between qualitative and quantitative methods for ethanol concentration calculation.

Sample	Ethanol conc. By using Quantitative Method	Ethanol conc. By Qualitative Method
2%	0.5	0.333
4%	0.5	0.7
6%	1.3	1.339
8%	2.7	1.529
10%	1.8	1.937

Results & Discussion

Figures (1: A-F) to (2: A-F) indicate to presence of relationship between sugar concentration and pH values in Ethanol and Biomass production from individual sugar (glucose, galactose) by individual yeast (*S.cerevisiae, C.kefy*-T, *C.kefyr*-S), the production of ethanol and biomass were gradually increased with increasing of sugar concentrations in media, under constant pH value, and the best production was at pH=5, in agreement with [16] who found relationship between sugar and (H+) concentrations in ethanol production from (maltodextrine) by *S. cerevisiae*, and pH(5, 5.5) were the best values for production. Result showed, at constant sugar concentration there was no significant differences in ethanol, biomass production at pH (4-6) values, and the best production was at pH average 5.0. This result was similar to those of [17], who found no high significant differences in ethanol production from (*Helianthus tuberosus*) plant by *S. cerevisiae* at (4.5-6.0) pH values, under constant sugar concentration, and the best production was at pH5. At all sugar concentrations used in this there is significant decreasing in production when pH value far away of (4-6). [18] Reported, that pH (4-6) values were suitable for growth and fermentative activity, while, fermentative activity was inhibited at (3-4) pH values, and the high pH values caused glycerol production instead of ethanol production. Same figures indicated a gradually increas in ethanol and biomass production, as sugar, concentration is increased, until the production reaches a maximum, then begin to decrease, this due to increase in osmotic pressure outside cell, because of high sugar concentration, hence ethanol accumulation inside cell, leading to decrease growth and fermentative activity, found the *S.cerevisiae* produced 4.6% ethanol, from 10% glucose concentration, this result in agreement with this study, which found, *S.cerevisiae* produced 5.1% ethanol, 4.4% Biomass from 10% glucose, at pH5.

Figures (1-C), (1-D), (1-E), (1-F), show, that best ethanol and biomass production by (*C.kefyr*-T) or (*C.kefyr*-S) was significantly at (6) % glucose and pH5, *C.kefyr*-T produced 2.5 % ethanol, 2.9% Biomass, while *C.kefyr*-S produced 2.2% ethanol, 2.5% biomass. This is due to the differences between these strains in glucose uptake rate, which is in agreement with [6], who found differences in glucose uptake rate even among strains of same yeast. Figures (1) (A-B) show, *S.cerevisiae* is the best producer of ethanol and biomass from glucose comparing with *C.kefyr* (S, T).

Figure 2(A) to (F) show, *C.kefyr* (T, S) is the best producer of ethanol and biomas from galactose than *S.cerevisiae*, but they are less galactose concentration tolerance than *S.cerevisiae*, who tolerant 6 % galactose concentration, while, *C.kefyr*, tolerant only 4%. *S.cerevisiae* produced 0.8% ethanol, 1.3% Biomass from 6% galactose while, *C.kefyr*-T produced 1.4% ethanol, 1.5% biomass, and *C.kefyr*-S produced 1.0% ethanol, and 1.2% Biomass, this may be due to. *S.cerevisiae* is greater galactose uptake rate through facilitated diffusion than *C.kefyr* (T&S), which is in agreement with, *S.cerevisiae* produced 0.6% ethanol, 0.2% biomass from 2% galactose, while *k. marxianus* produced 0.8% ethanol, 0.4% biomass during 48hr.

Figure 3 (A) to (D) show the best production was from 6% lactose under pH5, *C.kefyr*-T which produced 1.6% ethanol, 2.5% biomass, while *C.kefyr*-S produced 1.4% ethanol, 2.1% biomass, thus *C.kefyr*-T significant best ethanol and biomass production than *C.kefyr*-S. This is due to high activity of B-galactosidase production of *C.kefyr*-T comparing with *C.kefyr*-S. found (M) strain of lactose fermenting yeast (*K.marxianus*) was the best consuming lactose than other strains which were isolated from whey, dependency on number of produced B-galactosidase units by these strains. (Castillo *et al.*, 1982) found, the Maximum ethanol production from whey by *C.pseudotropicalis* was 0.38% from 5% Lactose and under 4.57pH value.

Ethanol production from mixed sugar by Individual yeast

Figure (4-A) shows, significant decreasing in *S.cerevisiae* ability for ethanol production from all concentration of equal ratio (glucose, galactose) mixture comparing with its producing from single sugar under pH5, *S.cerevisiae* produced half amount of ethanol production from glucose alone, this is due to inability of *S.cerevisiae* for galactose metabolism. The presence of glucose with equal amount of galactose in mixture causing fast decreasing in energy [20], reducing protein synthesis of lesion pathway which responsible for galactose metabolism in *S. cerevisiae*.

Figures(4-C)and (4-E) shows, significant dimension in an ability of *C.kefyr* (T&S) for ethanol production from mixture concentration comparing with their production from glucose alone, at pH5, but from mixture *C.kefyr* (T&S) produced more than half amounts of producing ethanol from glucose, due to their ability for glucose and part of galactose consumption in mixture during 48hr.

Figure (4- G), shows, although inability of *S. cerevisiae* for galactose uptake in mixture, but it's the best producer of ethanol from all mixture concentration comparing with *C.kefyr* (S&T) . found, although high ethanol production from pre hydrolyzed lactose to (glucose & galactose) by *S. cerevisiae*.

Figures (4-B) to (4-F) shows significantly decreasing in biomass production from all mixture concentration by individual yeast, comparing with production from individual sugars. This is due to presence of more than one sugar in media leading to a adaptation period with different dependency on sugar consumption rate by yeast found, yeast cell growth was observed in this period, and total producing biomass of *P. stipitis* was 13 g/g from (glucose & xylose) mixture, while 0.11 g/g from glucose alone.

Figure (4-H) shows, *S.cerevisiae* is the best production of Biomass from mixture sugar comparing with *C. ketyr* (S & T), this due to its ability for fast glucose consumption and continuous growth during 48 hr.

Figure (5-A), shows the inability of *S. cerevisiae* for direct ethanol production from all lactose concentrations, [2], stated, because of lacking cellular metabolism of lactose. *C.kefyr* (S & T) able to produce ethanol from all lactose concentration, (T) is the best producer than (S) at pH5, 35C° during 48 hr. *C. kefyr*- T produced 3. 3% ethanol, 2. 6% Biomass, while *C. ketyr*- S produced 2.9% ethanol, 2.4 % Biomass. This may be due to high efficiency of (T) for B- galactosidase enzyme unit's production in medium. There was a decreasing in the production by *C. Kefyr* (T & S) when Lactose concentration up to 8%, this is because of feedback effect of substrate concentration, hence, ethanol accumulation inside cell yeast [22].

Results in figure (5-B) show the ability of *S. cerevisae* for Biomass Production from all lactose concentration, and there is a closing between these values reaching to maximum production 2.8% when lactose concentration up to 8%, this may be due to increase of osmotic pressure on yeast cell because of high concentration of unconsumption lactose in medium [23] .

Ethanol production from mixed sugar by mixed yeast

Figure (6-A) , there is, significant increase in ethanol production from all equal ratio (glucose & galactose) mixture concentrations. Figure (6-B) shows significant decrease in Biomass production, when using the same mixed culture of (*C. kefyr*- T+ *C. kefyr*- S + *S. cerevisiae)* this may be due to absence of sequential fermentation of two sugars which start with glucose before galactose when using individual culture. Hence no adaptation period for secondary growth before galactose consumption found the presence of more than one monosaccharide in medium caused two growth periods of *S. cerevisiae*, according to yeast cell adaptation for each sugar. Results show that mixed culture of (*S. cerevisiae* & *C. kefyr*) was best ethanol and Biomass production from mixed sugar concentration, comparing with mixed culture of (*C. kefyr*- T + *C. kefyr*- S). This may be due to presence of glucose in media, and ability of *S. cerevisiae* for fast glucose uptake and conversion to ethanol, while galactose is consumed by *C. kefyr*. The mixed culture of (*C.Kefyr*- T + *S.cerevisiae*) was the best ethanol and biomass production than (*S. cerevisiae* + *C.kefyr*- S), (*C.kefyr*- T + *S. cerevisiae*) producing 8.7% ethanol, 5.0% biomass from 10% sugar mixture, this may be due to high galactose consumption rate of (T) comparing with (S).The production begin to decrease when mixture concentration up to 15%, thus because of inability of *C. kefyr* for high ethanol tolerance causing galactose accumulation in medium, hence, feedback of ethanol concentration on galactose utilization by (*C. Kefyr* + *S. cerevisiae*) and glucose and galactose metabolism by (*C. kefyr*- T+ *C.Kefyr*- S).

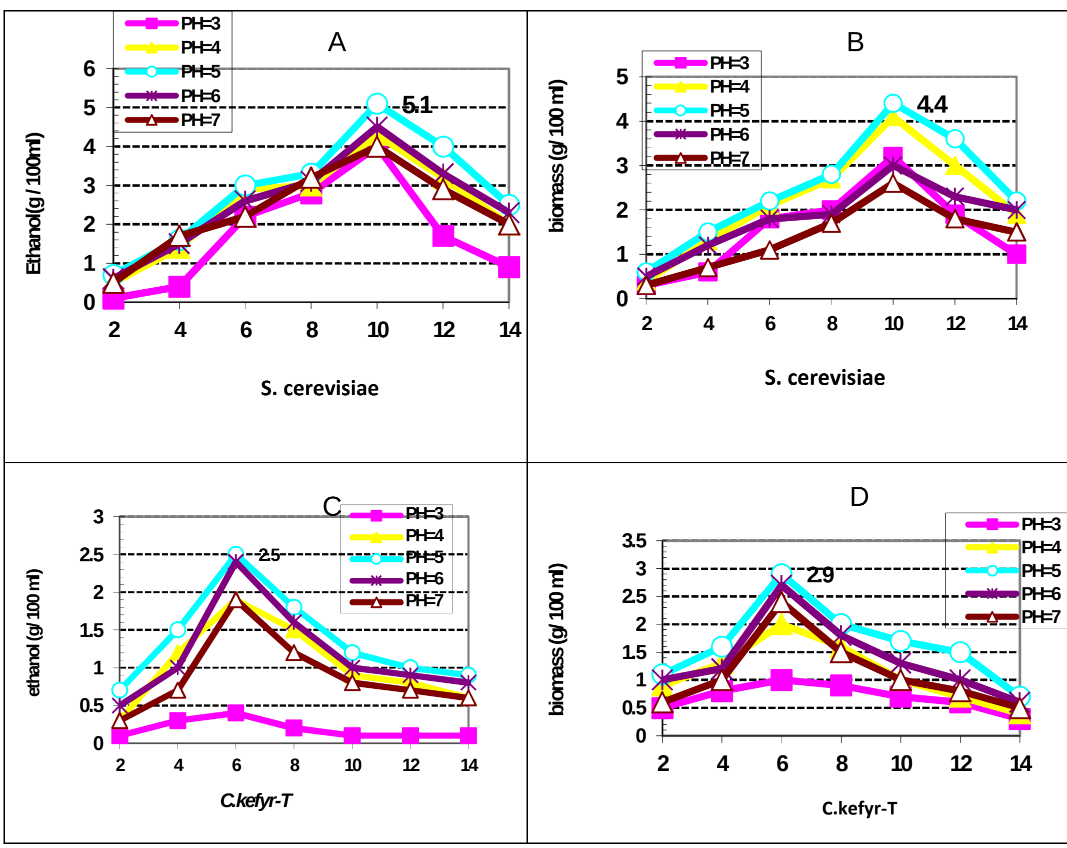
Figure (7-A) indicates a significant increase of ethanol production when using mixed culture of *S.cerevisiae* and (*C. kefyr*) (S, or T) than using individual (T&S). This may be due to presence of produced glucose from hydrolyzed lactose by (B- galactosidase) which produced by *C. kefyr*, and the ability of *S.cerevisae* for fast glucose consumption and conversion to ethanol, while, galactose was consumed by *C.ketyr* in mixed culture of (*S. cerevisiae* + *C. kefyr* . Ethanol production decreased when lactose concentration up to 8%, because of inability of *C.kefyr* for ethanol concentration tolerance, found, that mixed culture of (*k.fragilis* + *Z. mobilis*) produced (5. 5) % ethanol from (20) % lactose.

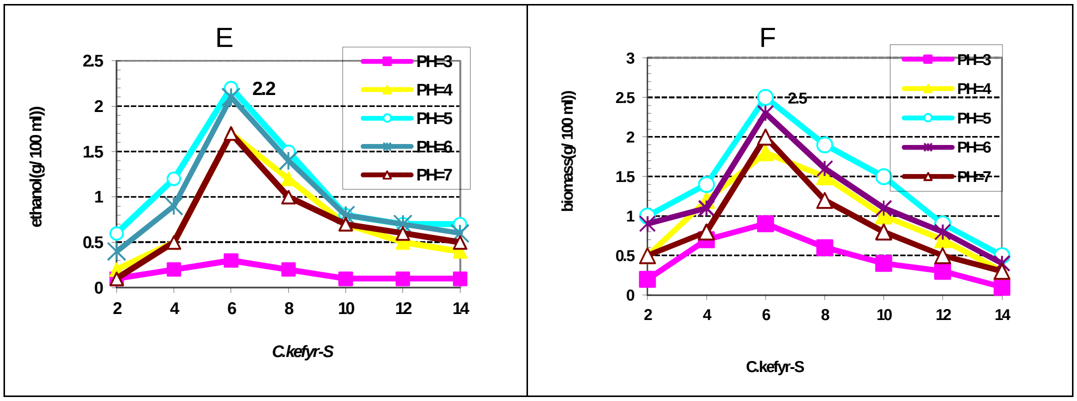
Results in figure (7-B) indicate a significant decrease in ethanol and Biomass production, when using mixed culture of *(S. cerevisiae* + *C. kefyr*- S). This may be due to less soluble oxygen in media, and because of *S.cerevisiae*, which has an effect on *C. kefyr* growth and, it's B- galactosidase production activity .

Effect of bioreactor on ethanol production

A different periods time of (24, 36, 48, 60) hours during the fermentation were presented. The initial sugar concentration was 10% and the mixed yeast culture were (*C. kefyr*- T + *S. cerevisiae*) in all cases, because they gave the best ethanol% as shown in figure (6-A) . The data in figure (8) indicated a significant additional ethanol yield by increasing period during fermentation. The highest ethanol production during 48 h was 15.5% by bioreactor that equal nearly double fold of 8.7% ethanol produced by still culture at the same conditions (fig.6-A). Other ethanol percentages remained at low levels within other periods. This result appeared higher than the value achieved by who stated 14% ethanol produced by baker's yeast.

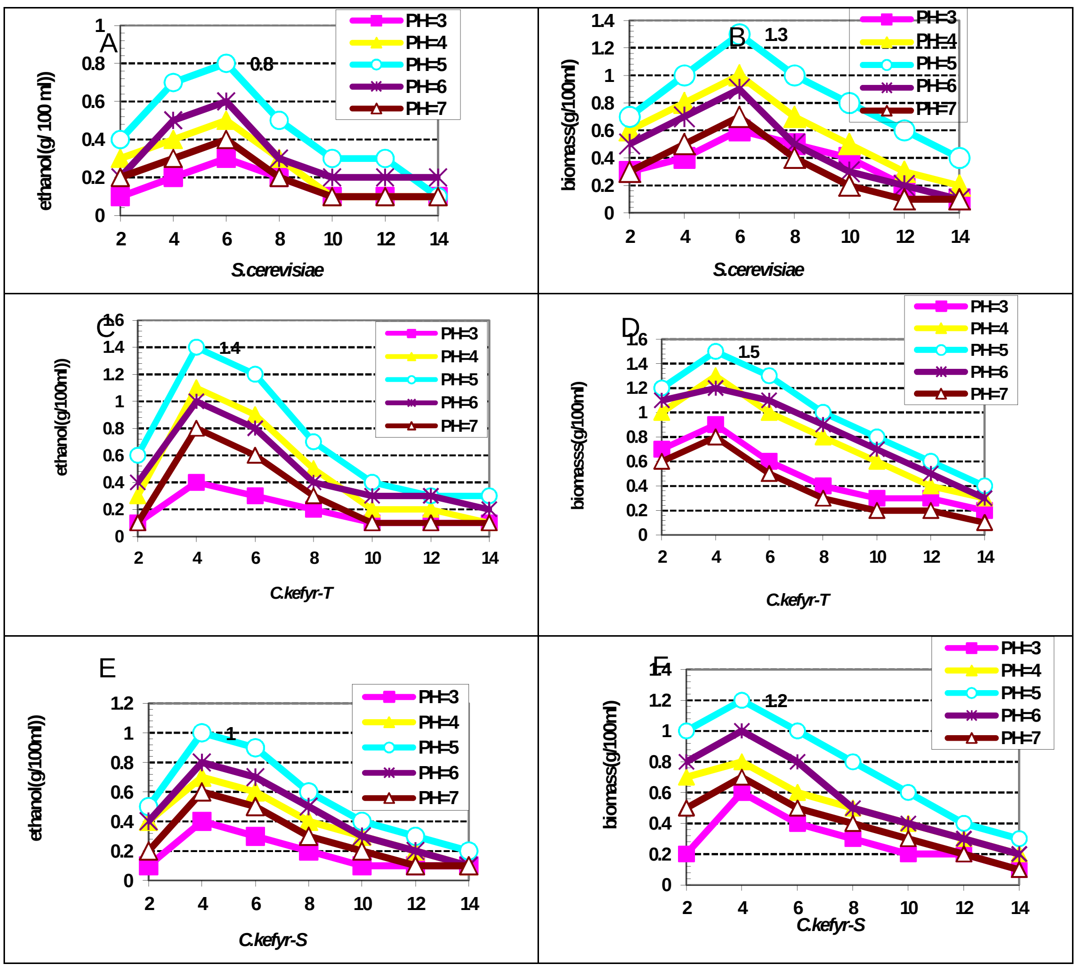
biomass16% in figure (8) gained from the same conditions in the bioreactor process. The mixed sugars concentration of 10% was a suitable concentration in bioreactor fermentation for ethanol production because it appears in this study that low concentrations of sugar results in low ethanol concentration during the fermentation. On the other hand, a very high sugar concentration can result in high stress and inhibition of microorganisms by either the sugars or ethanol. The results in this study revealed that the cultivation conditions of growth pH, fermentation period, single & mixed yeast culture, single & mixed sugar concentration and bioreactor fermentation process affected not only the fermentation rate, but also biomass formation and ethanol production. However, the overall results showed that mixed sugar of (glucose + galactose) gave the highest ethanol productivity followed by glucose and galactose, respectively by mixed yeasts (*C. kefyr*- T + *S. cerevisiae*). Physiologically *S. cerevisiae* would utilize glucose, whereas *C. kefyr*- T would utilize galactose. This could be attributed to the fact that the enzyme system complementary is more in two yeasts mixture compared to one yeast alone. These phenomena so needed to be studied in a further fermentation investigation in order that the production of higher levels of ethanol from any fermentable mixed substrate can be utilized by other mixed microorganisms.





Glucose% Glucose%

Figure (1):effect of glucose % & pH on ethanol and biomass production by individual yeast at 35 C for 48 h.



Galactose% Galactose%

Figure(2):effect of galactose % & pH on ethanol and biomass production by individual yeast at 35 C for 48 h.

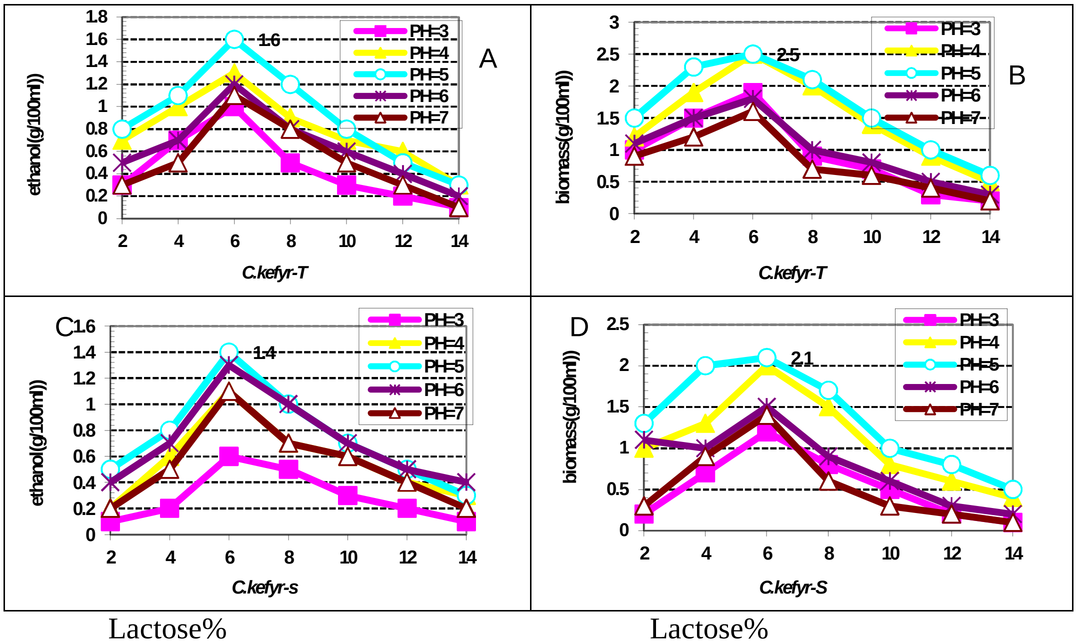
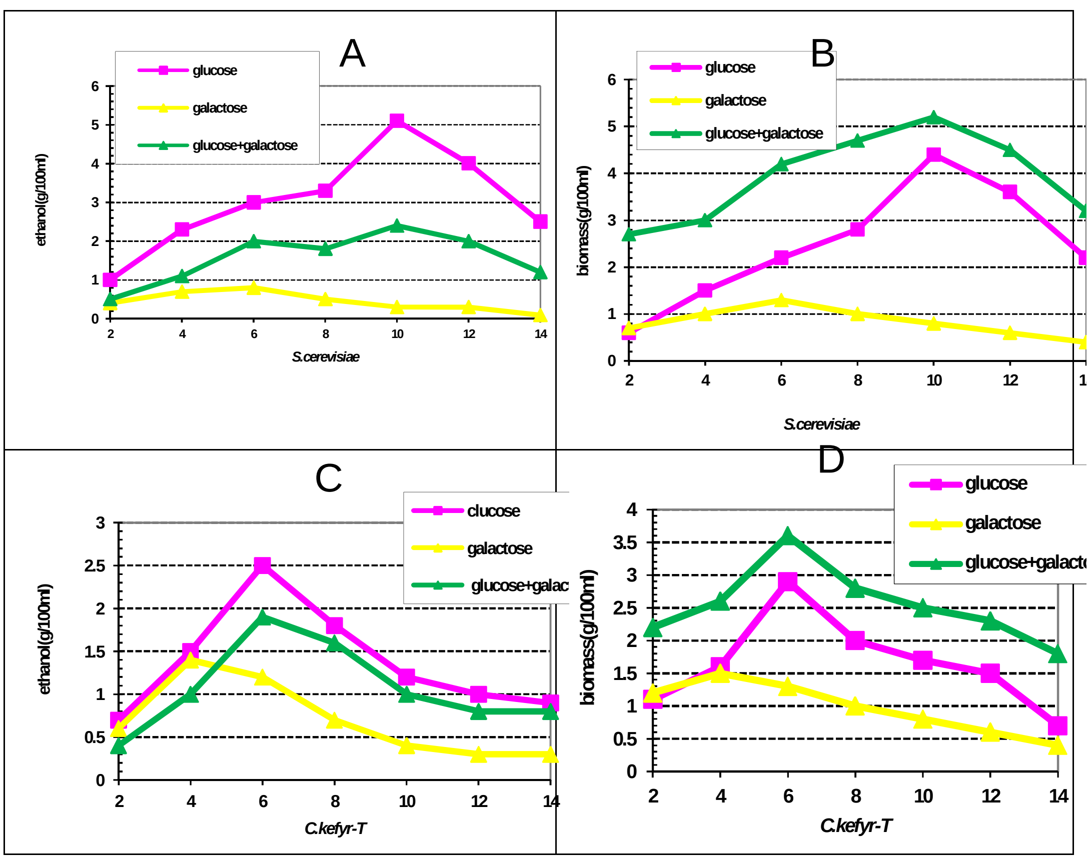
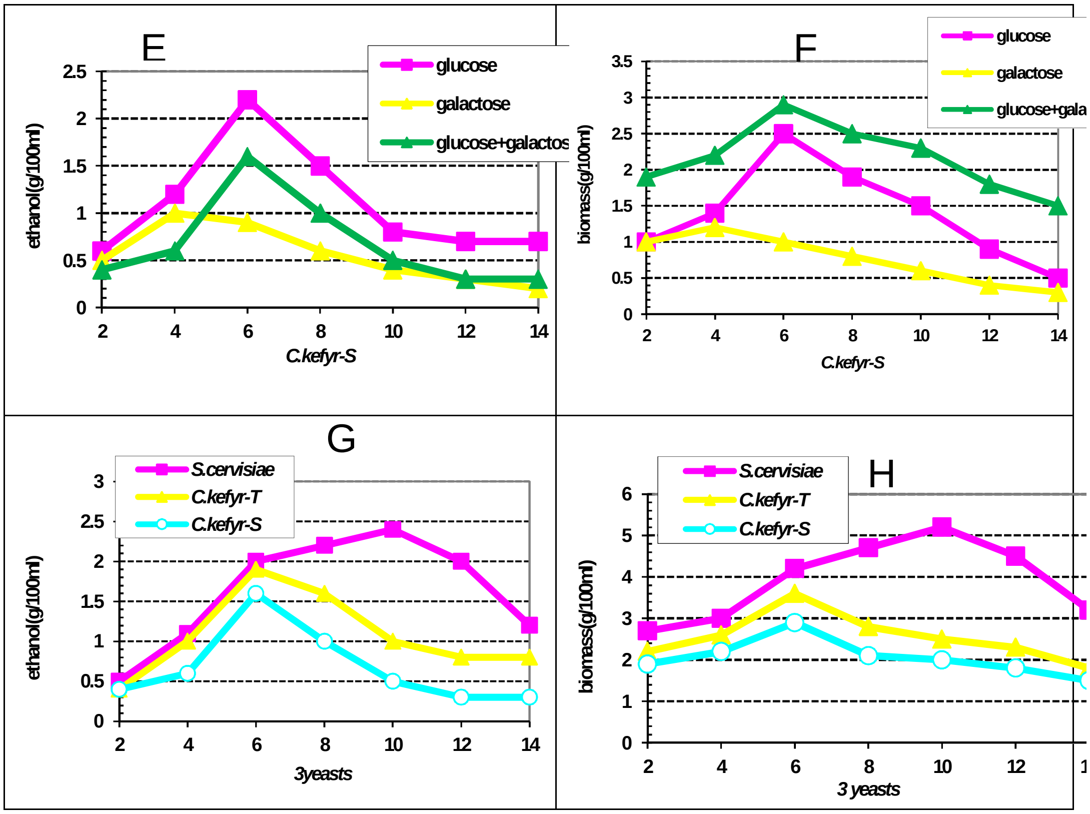


Figure (3):effect of lactose % & pH on ethanol and biomass production by individual Yeast at 35 C for 48 h.





Mixture sugars Mixture sugars

Figure (4): effect of (glucose&galactose) mixture% in ethanol and biomass production at pH5,35c for two days

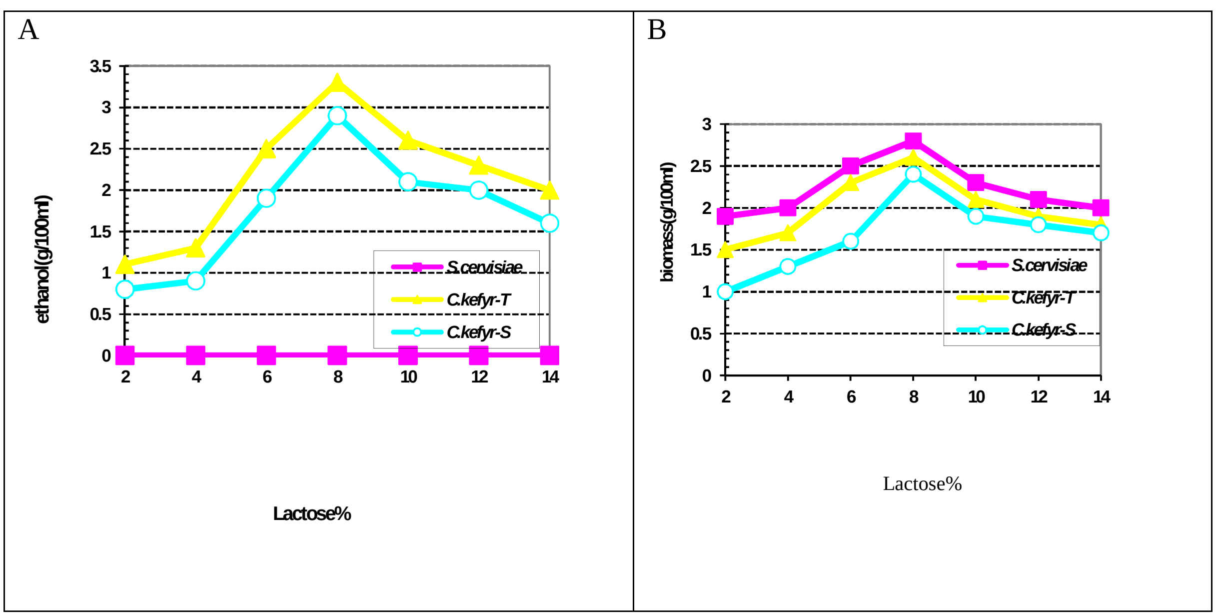


Figure (5): effect of lactose % on ethanol and biomass production by individual and mixed yeast growth at pH5, 35C for 48h.

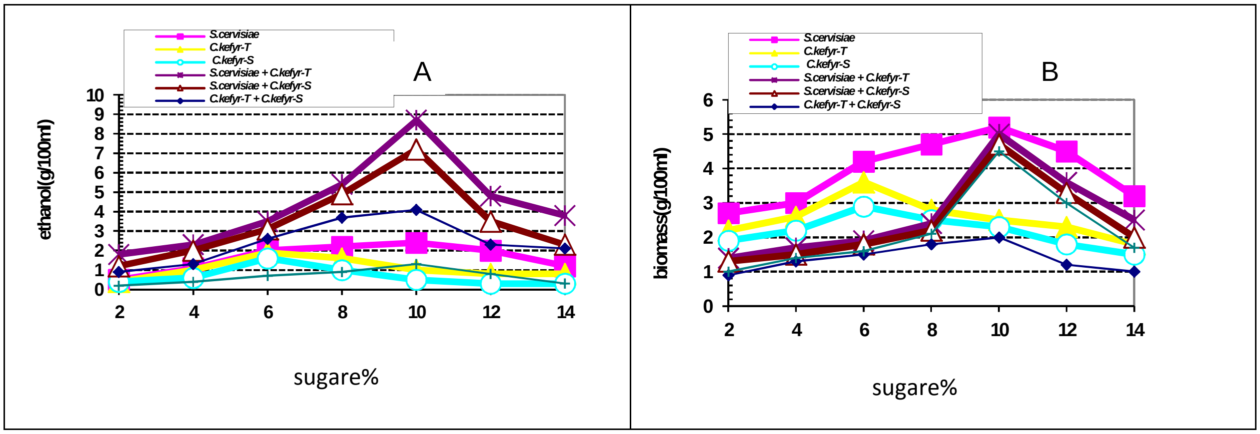


Figure (6): effect of (glucose&galactose) mixture% on ethanol and biomass production at pH5, 35C by individual and mixed yeast growth

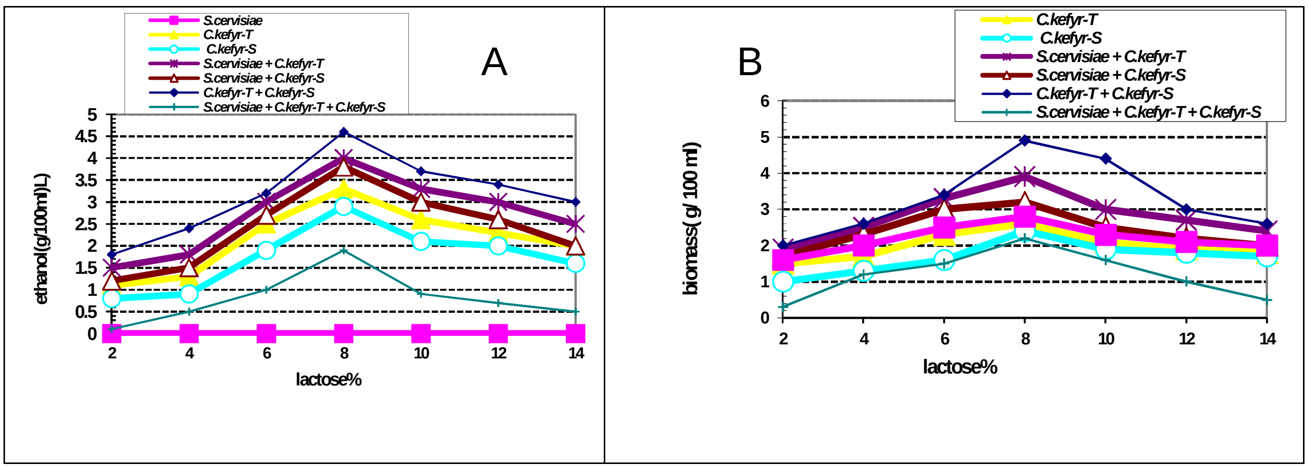


Figure (7): effect of lactose conc% in ethanol and biomass production by individual and mixed yeast growth at pH5, 35C for two days

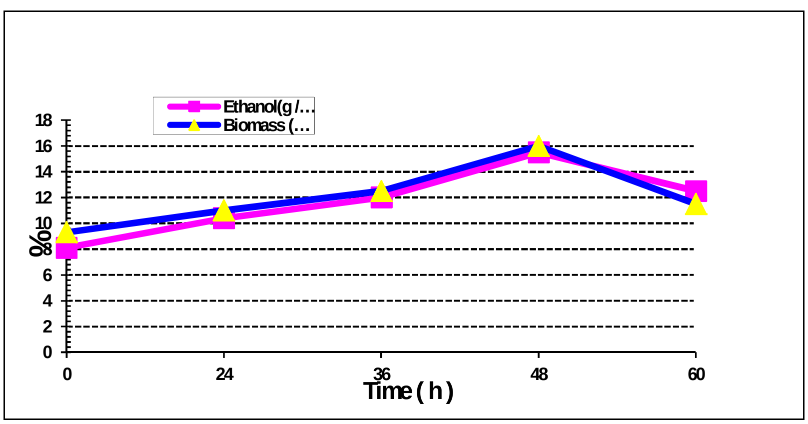


Figure (8): effect of bioreactor process on ethanol and biomass production from lactose conc. 8.0% by mixed yeast)*C.kefyr*-T + *C.kefyr*-S) growing at pH5, 35C for different periods (h).

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