Studies of Bioethanol Production from Some Carbohydrate Sources by Gram Positive Bacteria

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Abstract: Fermentation is a process by which large organic molecules are broken down in to simpler molecules as the result of the activity of microorganisms. Bioethanol is produced by the activity of some bacteria, and yeast, and their actions on substrates containing carbohydrates. Biofuels are a wide range of fuels that are in some way derived from biomass. Biofuels are gaining increased public and scientific attention, due to factors such as oil price spikes and the need for increased energy security.
It was observed that nutrients play a vital role in the process of fermentation. During the present study, seven bacteria were isolated from rotten fruits, out of which four bacteria (A, B, X and Y) all bacteria were gram positive and rod-shaped. They were able to ferment carbohydrate and produce bioethanol. The different substrates barley, oat, maize and sugar beet were used for bioethanol production. During the present investigation, the effects of different macro- and micro-nutrients on bioethanol production were also assessed.
It was observed that after supplementation of 5 ml and 10 ml of macronutrients (carbon, nitrogen, sulfur and phosphorus) in all four substrate solutions (barley, oat, maize and sugar beet), the barley had produced the most bioethanol. Thus, it was observed that macronutrients had a relevant effect on bioethanol production. On the contrary, large amounts of production were not seen when micronutrients (aluminum, copper, chromium and zinc) were added in pure form.

Key words: Bioethanol, Fermentation, Macronutrient, Micronutrient and Bacteria.

1. Introduction

Biotechnology is a discipline that studies with the use of living organisms or their products in large-scale industrial process. Microbial biotechnology is that aspect of biotechnology that deals with processes involving microorganisms. We are living in an era of microbial technology. Due to the advent of gene technology, a completely new approach to microbial biotechnology is introduced in which the microorganism is engineered to produce a substance [1]. The term fermentation is derived from the Latin word 'fervere', which means 'to boil' and it describes the action of yeast on extracts of fruits or malted grains, which appears as if it is boiling. The boiling appearance is due to the production of carbon dioxide bubbles caused by an aerobic catabolism of the sugars present in the extract. Fermentation utilizes organic substances and organic electron acceptors and reduced organic substances are produced as end-products. ATP is obtained by substances [2]. According to the theory of fermentation, if starting material is carbohydrate (polysaccharide) it is converted in to simple sugars (monosaccharide) such as glucose. Those sugars are then converted to alcohol and carbon dioxide. A number of enzymes are needed to carry out the sequence of reactions, the most important of which is Zymase found in yeast cells. Fermentation has number of commercial applications. The selection of right bacteria and the right conditions is an art in the process of food production, so that the products have the desired flavors.

Sugarcane is the world's largest source for microbial production of bioethanol [3]. Today, raw materials used in the manufacture of bioethanol by fermentation are classified as sugars, starches and cellulosic materials [4]. Ethanol is more commonly obtained by ethylene hydration the reaction of ethylene with water in the presence of phosphoric acid [5]. One of the most successful commercial applications of fermentation has been the production of ethyl alcohol for use in gasohol. Gasohol is a mixture of about 90% gasoline and 10% alcohol. The use of gasohol increases the availability of a non-renewable resource. Biologically ethanol is produced by involvement of some bacteria, yeast and their action on substrate containing carbohydrate and it is known as bioethanol. Bacteria *Zymomonas mobilis* are the most widely used microorganism for ethanol production [6]. Bioethanol has been trusted as an alternate fuel for the future. The lack of industrially suitable microorganisms for converting biomass in to fuel ethanol has traditionally been cited as a major technical roadblock to developing a bioethanol industry. In the last two decades, numerous microorganisms have been engineered to selectively produced bioethanol. The construction of *Escherichia coli* strains to produce ethanol selectively was one of the first successful applications of metabolic engineering [7]. *Zymomonas mobilis* yields high ethanol and can tolerate high ethanol concentration but it is not well suited for biomass conversion because it ferments only glucose, fructose and sucrose. Nevertheless in last decades it has been engineered and is now made capable of fermenting xylose and arabinose too. The first recombinant strain was engineered to ferment xylose [8]. Some gram-positive bacteria *Clostridium cellulolyticum, Lactobacillus casei* have been engineered for bioethanol production [9].

Bioethanol is based on renewable resources, which help the world to secure its future supply of energy by reducing its dependency on fossil fuels. Bioethanol has a number of advantages over conventional fuels because it is produced from renewable resources. Bioethanol is high-octane fuel and has replaced lead as an octane enhancer in petrol. By blending ethanol with gasoline, it can oxygenate the fuel mixture so that it may burn more completely and reduce polluting emission. In bioethanol production process only renewable energy sources are used and no net carbon dioxide is added to atmosphere during use of bioethanol so bioethanol an environmentally beneficial energy source.

The present work was carried out for the production of bioethanol from some carbohydrate source that is grains (barley, oat, maize) and sugar beet, which are wasted in godowns and croplands. Bioethanol is renewable source of energy and can overcome the problem of energy crisis in near future.

2. Materials and Methods

Ethanol is an important industrial compound that is produced biologically as well as chemically. Biologically, it is produced by some bacteria and fungi by their fermentation and using carbohydrates substrate corn, sugar beet, sugarcane,

potatoes, oat, wheat etc. As reported *Zymomonas mobilis* do most production of ethanol. During the present investigation, bacteria were isolated from rotten fruits and the substrates used in this investigation were barley, oat, maize and sugar beet. The following steps were followed for the objectives:

2.1 Collection of Samples

The rotten fruits was collected from waste and its juice was poured on nutrient agar medium (NAM) for culture of bacteria and kept it for 24 hours at 37°C for growth of bacterial colony.

2.2 Isolation of Bacteria

Different bacterial colonies were grown on nutrient agar medium. The grown cultures were recultured as pure culture through the streak plate method in Nutrient Agar Medium (NAM).

Composition of NAM [10] :		
Peptone	-	05 g
Sodium chloride	-	05 g
Beef extract	-	03 g
Agar	-	15 g
Distilled water	-	1000 ml
pH	-	7.3

2.3 Selection of Bioethanol Producing Bacteria by Process of Fermentation

The process of isolating bacteria, obtained seven types of bacteria of unidentified genera, which are characterized by their cultural and physiological characteristics (further identification is going on). By fermentation test we had confirmed that whether they are ethanol-producing bacteria or not. Fermentation test is standard protocol for fermentation of carbohydrate by bacteria [11]. In this method fermentation broth was prepared, composition of broth is peptone (10 g), sodium chloride (15 g), carbohydrate (5 g), phenol red (0.018 g) and distilled water (1000 ml).

- In this test, a fermentation broth was prepared and in test tube and durum's tube was inverted in test tube and broth was autoclaved.
- All test bacteria were inoculated in it and kept it for incubation for 48 hours at 37°C.
- The red color of the broth converted to yellow and the formation of gas bubbles in a durum's tube showed a positive test.

2.4 Cultural and Physiological Characteristics of Four Selected Bacteria

In the fermentation test, four out of seven bacteria gave positive test. They were named as A, B, X and Y. These four bacteria, that gave positive test were cultured and preserved for further study. Later on, their physiological study was conducted. There are several staining methods available to study the properties of microorganisms. Cultural characteristics were observed by colony characteristics, but the shape study and physiological characteristics were done by gram staining, acid fast staining, endospore staining and motility.

2.5 Raw Materials to Be Used

The fermentation process occurs in materials that contains carbohydrates, which are converted in-to ethanol. The carbohydrate-containing substrate converted in-to sugar and then to ethanol. In our study, we have taken barley, oat, maize and sugar beet as raw materials. Their approximate nutrient composition is the following:

Nutritional value per 100 g (*en.wikipedia.org*)

Sugar beet		
Carbohydrate	-	9.56 g
Fat	-	0.17 g
Protein	-	1.61 g
Dietary fibers	-	2.80 g
Oat		
Carbohydrate	-	66.0 g
Fat	-	06.0 g
Protein	-	17.0 g
Dietary fibers	-	11.0 g
Barley		
Carbohydrate	-	77.7 g
Fat	-	01.2 g
Protein	-	09.9 g
Dietary fibers	-	15.6 g
Maize		
Carbohydrate	-	19.02 g
Fat	-	01.18 g
Protein	-	03.22 g
Dietary fibers	-	02.70 g

2.6 Method of Bioethanol Production

Different grains (maize, barley, and oat) and sugar beet were taken, 50 g in 500 ml of distilled water and submerged in water overnight. Crushing was done in the same water.

- All substrates were crushed properly by mortal pestle.
- All juices were autoclaved.
- Selected bacteria were inoculated in it and the high production given bacteria was inoculated in each substrate, Y in barley and maize, B in oat and A in sugar beet.
- It was kept in incubator shaker for 24 hours at 37°C [12].

2.7 Estimation of Bioethanol

2.7.1 Qualitative estimation: Examine the bioethanol production by Jones reagent [$K_2C_{r2}O_7+H_2SO_4$] [13]. 1 ml of $K_2C_{r2}O_7$ (2%), 5 ml of H_2SO_4 (concentrated) and 3 ml of sample was added. Ethanol was oxidized in-to acetic acid with potassium dichromate in the presence of sulfuric acid and it gave blue green color. Green color indicates positive test [14].

2.7.2 Quantitative estimation: Substrate solution was distilled in alcohol distillation unit for quantitative estimation of bioethanol. For the quantity estimation of bioethanol, the standard curve was formed by using the pure ethanol with the series of 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml, 3.0 ml, 3.5 ml and 4.0 ml, that were oxidized by Jones reagent [$K_2C_{r2}O_7+H_2SO_4$]. Optical density (OD) was measured through spectrophotometer at 600 nm [15].

2.8 Effect of Different Macronutrients on Bioethanol Production

Macronutrients were nitrogen in the form of urea, carbon in the form of glucose, sulfur in the form of ammonium sulfate and phosphorus in the form of ammonium phosphate. All macronutrients were made 1% (1 g of macronutrient in 100 ml of distilled water) solution and 5 ml and 10 ml amount were added to the substrate solution. It was inoculated with selected bacteria after autoclaving and was kept in incubation for 48 hours at 37°C in an incubator shaker.

2.9 Effects of micronutrients on bioethanol production

Metals (zinc, aluminum, copper and chromium) were taken in powder form 1% (1 g of nutrient in 100 ml of distilled water) and the stock of micronutrients was prepared. However, pure metals did not dissolve, even then the solution was well shaken for a considerable time to find the effect of metals. The most successful bioethanol producing bacteria were used with

different nutrients. All four substrates were inoculated with 0.5 ml solution of different metals in pure form; metals were zinc, aluminum, copper and chromium. After autoclaving and bacterial inoculation it was kept for incubation for 48 hours at 37°C in incubator shaker.

3. Results and Discussion

The present study was done on barley, oat, maize and sugar beet. The production of bioethanol was done by gram-positive bacteria and their action on substrates (barley, oat, maize and sugar beet). The results of bioethanol production are described below:

The seven types of bacteria obtained from rotten fruits were cultured by streak plate method. These bacteria were subjected to fermentation test in which four out of seven gave the positive test. They were unidentified and named as A, B, X and Y. It was confirmed whether they were ethanol producing bacteria or fermentative bacteria by the following fermentation test. Bacteria when inoculated in fermentation broth it convert red color of broth to yellow color due to production of acid and formation of air bubble due to production of gas. Bacteria A and Y had a creamish colored colony and rough texture. The colony of bacteria B was creamish yellow color and of rough texture. The appearance of X was white colored colony and rough texture. All bacteria were rod shaped. Physiological characteristics of all bacteria were observed. They all were gram positive, acid-fast negative, non- motile and endospore bearing. Gram staining is an important method to classify the bacteria in-to two large groups: gram positive and gram negative by the chemical and physical properties of their cell walls. Senthilkumar and Gunasekaran studied that some gram-positive bacteria *Clostridium cellulolyticum, Lactobacillus casei* have been engineered for bioethanol production. Dien *et al.* worked on Gram-negative bacteria *Escherichia coli, Klebsiella oxytoca,* and *Zymomonas mobilis. E. coli* and *K. oxytoca* are naturally able to use a wide spectrum of sugars, and work has concentrated on engineering these strains to produce ethanol selectively. *Z. mobilis* produces ethanol at high yields, but ferments only glucose and fructose [16]. Jadhav *et al*. worked on bioethanol production by four gram-positive bacteria on substrate Mahua flowers [17]**.** Talarico *et al.* constructed an operon for expression of ethanol production in gram-positive bacteria [18].

Schwietzke *et al.* worked on potential of fuel ethanol as an additional source of product based on utilization of the cellulosic portions of maize and in particular the pericarp, cobs, stalks and leaves of the corn plant [19]. Thomas and Ingledew used hulled and hull-less oats and fermented at 20°C with active dry yeast to produce bioethanol for fuel alcohol production [20]. Kim *et al.* worked on barley hull, a lignocellulosic biomass, was pretreated using aqueous ammonia, to be converted into ethanol [21].

Bioethanol was produced by fermentation and distilled by distillation unit and amount of ethanol was calculated by standard curve. Qualitative estimation was done by Jones reagent [$K_2C_{r2}O_7+H_2SO_4$] and quantitative estimation was done by using standard curve of ethanol and spectrophotometeric analysis.

The production of bioethanol at 37°C was also studied. In barley, bacteria A gave 3.00 ml, B 3.30 ml, X 1.50 ml and Y gave 3.40 ml of bioethanol. In substrate oat bacteria A gave 1.50 ml, B 2.02 ml, X 0.83 ml and Y gave 1.26 ml of bioethanol. In substrate maize bacteria A gave 0.54 ml, B 0.46 ml, X 0.84 ml and by Y 2.08 ml of bioethanol was produced. In substrate sugar beet A gave 1.14 ml, B 0.57 ml, X 0.47 ml and by Y 0.39 ml of bioethanol was produced. In this study the amount of bioethanol was maximum in barley which is 3.40 ml by bacteria Y, then in maize 2.08 ml by bacteria Y again, and then oat 2.02 ml by bacteria B and in sugar beet it was observed 1.14 ml by bacteria A. All these highest producing bacteria were used for specific substrate to see the effect of macro and micronutrients (Table 1).

Table 1. Amounts of bioethanol at 37°C.

Bacteria Sugar beet	Barley	Oat	Maize
A **1.14 ml**	3.00 ml	1.50 ml	0.54 ml
B 0.57 ml	3.30 ml	**2.02 ml**	0.46 ml
X 0.47 ml	1.50 ml	0.83 ml	0.84 ml
Y 0.39 ml	**3.40 ml**	1.26 ml	**2.08 ml**

Different macronutrients such as nitrogen (urea), carbon (glucose), phosphorus (ammonium phosphate) and sulfur (ammonium sulfate) were supplied in amounts of 5 ml and 10 ml in substrates. When 5 ml nitrogen compound was added to barley, it produced 5.10 ml whereas in oat gave 0.80 ml, maize 2.50 ml and sugar beet 0.39 ml of bioethanol. When 10 ml of nitrogen compound was added in different substrates, the bioethanol production in barley was 2.90 ml, whereas in oat it was 0.41 ml, maize 2.09 ml and in sugar beet 0.28 ml. When 5 ml of carbon compound was added to different substrates, the bioethanol production in barley was 1.04 ml, whereas in oat 0.68 ml, in maize 0.58 ml and in sugar beet 0.34 ml of bioethanol was produced. When 10 ml carbon compound was supplied in different substrates, the bioethanol production in barley was 0.89 ml, whereas in oat 0.53 ml, in maize 0.48 ml and in sugar beet 0.28 ml. When 5 ml sulfur compound was added to substrates, the bioethanol production in barley was 1.30 ml, whereas in oat 0.60 ml, in maize 1.04 ml and in sugar beet 0.46 ml. When 10 ml of sulfur compound was added in different substrate, the bioethanol production in barley was 1.50 ml, whereas, in oat 0.94 ml, in maize 1.10 ml and in sugar beet 0.62 ml. When 5 ml of phosphorus compound was added to different substrates, the bioethanol production was in barley 1.20 ml, in oat 0.60 ml, in maize 0.76 ml and in sugar beet 0.55 ml. When 10 ml of phosphorus compound was added in different substrate the bioethanol production in barley was 0.87 ml, whereas in oat 0.55 ml, in maize 0.66 ml and sugar beet 0.51 ml. Supplementation of different macronutrients clearly showed that only nitrogen had relevant effect on the bioethanol production and it was highest in barley that was 5.10 ml and next in maize which was 2.50 ml (Tables 2 and 3).

Table 2. Effects of all macronutrients (N, C, S, and P) in amounts of 5ml on bioethanol production.

Substrate	Nitrogen	Carbon	Sulfur	Phosphorus
Barley	**5.10 ml**	1.04 ml	1.30 ml	1.20 ml
Oat	0.80 ml	0.68 ml	0.60 ml	0.60 ml
Maize	2.50 ml	0.58 ml	1.04 ml	0.76 ml
Sugar beet	0.39 ml	0.34 ml	0.46 ml	0.55 ml

Table 3. Effects of all macronutrients (N, C, S, and P) in amounts of 10 ml on bioethanol production.

Substrate	Nitrogen	Carbon	Sulfur	Phosphorus
Barley	**2.90 ml**	0.89 ml	1.50 ml	0.87 ml
Oat	0.41 ml	0.53 ml	0.94 ml	0.55 ml
Maize	2.09 ml	0.48 ml	1.10 ml	0.66 ml
Sugar beet	0.28 ml	0.28 ml	0.62 ml	0.51 ml

The supplementation of micronutrients like alumina, copper zinc and chromium showed no significant effect on the production of bioethanol (Table 4). Lapaiboon *et al.* worked on ethanol production from sweet sorghum juice by *Saccharomyces cerevisiae* NP01 was investigated under very high gravity fermentation and various carbon adjuncts and nitrogen sources [22]. Pradeep and Reddy worked on high gravity fermentation of sugarcane molasses to produce ethanol and effect of nutrients on production [23]. Ayse and Sedat worked on ethanol production and studied the effect of zinc sulfate [24]. It is possible that the production may be enhanced by metals in their compound form.

Table 4. Effects of different micronutrients (Al, Cu, Cr, and Zn) in amounts of 0.5 ml on bioethanol production.

Substrate	Aluminum	Copper	Chromium	Zinc
Barley	1.14 ml	0.93 ml	1.17 ml	0.39 ml
Oat	1.11 ml	0.04 ml	0.06 ml	0.04 ml
Maize	0.46 ml	0.12 ml	0.69 ml	0.39 ml
Sugar beet	0.04 ml	0.02 ml	0.09 ml	0.08 ml

4. Conclusion

The findings of the present study can show that bioethanol can be a promising fuel and can overcome the energy crisis in the future. The cereals and sugar beet, which are wasted in croplands, can be used to produce bioethanol. The application of different macro- and micro-nutrients can improve production of bioethanol. Today, the world is facing the problem of health, energy and environment, all of which can be solved by bioethanol because bioethanol is eco-friendly, less polluting and can be a useful alternative source of energy.

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