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Studies on Bio-ethanol Production from Orange Peels using *Bacillus subtilis*

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Abstract: ***A large amount of waste material is coming from food industries and disposal problems are leading to pollution. In the present study, the capability of isolated and identified Bacillus subtilis to produce bio-ethanol from orange peels was investigated. The process parameters were optimized to improve bio ethanol production. From the studies, it was found that the maximum amounts of ethanol produced using orange peels at concentration of 8%. 67.20 g/L bio-ethanol was produced with the addition of 2% sucrose by Bacillus subtilis. The maximum amount of bio-ethanol was produced as 75.20g/L using Bacillus subtilis by adding ammonium chloride to the medium at 4.0g/L. The influenced process parameters such as inoculum concentration, pH, temperature and incubation time were found to be optimum at 10%, 7, 35°C, 72 h respectively. In the presence of Bacillus subtilis the bio-ethanol production was found to be 92.25% at these optimum conditions.***

Keywords: Bioethanol, orange peels, biomass waste, fermentation, optimization, *Bacillus subtilus*

1. Introduction

Global energy consumption and dependence on non-renewable energy resources has increased the cost of transportation and highlighted the environmental problems by affecting the durability of ecosystems, global climate as well as oil reserves that result in greenhouse effect. Hence, the need for an alternative energy source arises. A new wave of technologies is on the edge of producing energy that is clean, renewable, and most importantly economical. Bioethanol as a clean and renewable fuel has gained more attention as a substitute to fossil fuel [1]. Lignocellulosic substrates shown prominent attention as raw material for biofuel production due to immense amount of supply compared to other biomass; they are relatively low cost, sustainable in supply and without food conflict [2]. For sustainable production of bioethanol, lignocellulosic biomass has been selected as it is available in the form of hardwood and softwood such as agricultural and forest silts, crops and herbaceous materials in large quantities.

Enormous quantities of agro-industrial waste residues are generated throughout the world from processing raw agriculture materials for foods. These wastes and their disposal have become an environmental concern especially when they are biodegradable to useful goods and services [3]. Cellulolytic wastes from agricultural practices can be used to produce important compounds such as alcohol thereby assisting in controlling environmental pollution [4].

Most of us who eat oranges, normally throw out the peels, but what many of us are unaware of is that these peels are loaded with highly nutritious compounds that are beneficial for our health. Orange peels contain more than 60 types of flavonoids and over 170 different types of phytonutrients, in addition to being rich in various pectins, vitamins, minerals and fibre. Orange peels belong to this group of valuable biomass wastes [5]. The peel contains various carbohydrate polymers, which make it an interesting choice for production of metabolites such as ethanol by appropriate microorganisms. Several researchers [6-9] proposed the citrus waste for different applications like production of pectin, flavonoid, fiber, and animal feed production. However, a large amount of this waste is still dumped every year [10], which causes both economic and environmental problems such as high transportation cost, lack of dumping site, and accumulation of high organic content material [11]. Therefore, more effective and sustainable alternatives for using orange peel wastes such as biogas are highly desirable.

The present study was carried out to investigate the production of bio-ethanol by *B. subtilus* utilizing orange peels waste and to optimize medium components and culture conditions to improve bio-ethanol production.

2. Materials and Methods

Pretreatment and acid hydrolysis of orange peels

The waste from orange juice process and contained 21.3% total solid (TS). The oranges samples were procured from the local market of Warangal town. It was then chopped prior to pretreatment process. Orange peels, an agro-industrial waste, were used in this study as a substrate for bio-ethanol production. Pretreatment of the wastes by leaching was conducted in Erlenmeyer flasks. Four different solvents including hexane, diethyl ether, dichloromethane, and ethyl acetate were used. These chemicals are toxic and/or inflammable and should be treated properly. In the experiment to select the solvent, each solvent was added to the orange peel with orange peel waste and hexane ratio of 1 : 4. The mixture of solvent and orange peel was shaken vigorously for 10 minutes followed by incubation for 20 minute at room temperature. In the optimization of pretreatment study, hexane was used as a solvent. Two levels of four parameters including temperature (20°C and 40°C), time (10 min and 300 min), orange peel wastes and hexane ratio (1 : 2 and 1 : 12), and the wastes size (chopped) were selected. Forty grams of the wastes was dissolved with a certain amount of hexane in the flasks, followed by shaking vigorously for a determined period of time. After the settlement, the extracts were removed from residuals by vacuum filtration. The peels were separated and stored frozen at -20°C. The dry content of orange peel was 18.7%

and determined by drying the peels at 110°C for 48 h. Before hydrolysis, the peels were thawed and ground with a food homogenizer to less than 2 mm in diameter.

Microorganism

The bacterial strain *Bacillus subtilus* was isolated from wastewater of NIT Warangal waste water treatment plant [12]. At a pH of 6.8, stock cultures were grown on nutrient agar slopes and inoculated slopes were incubated at 30°C for 24 h, subsequently at 4°C in sealed universals. By transfer on to fresh agar medium, they were periodically sub-cultured.

Fermentation process and bio-ethanol production

In a 250 ml flask, one loop of cells of the bacterial strain was transferred to 50 ml of nutrient broth medium prepared with distilled water and aerobically cultivated for 24 h. The centrifugation was done 10000 x g for 10 min for the cell culture and washed three times with sterile physio-logical saline (NaCl 8.5 g/l). The concentration of 0.01 g/mL was obtained when cells were suspended in the same solution. The cells were transferred to 50 ml of minimal medium (MM) in a 250 ml Erlenmeyer flask containing: ammonium sulfate at 2 g/L, magnesium sulfate at 0.2 g/L, dibasic potassium phosphate at 0.7g/L, monosodium phosphate at 0.3 g/L, and 5 mL of a micronutrient solution [13]. Thiamine stock solution (0.1% w/v) was filter sterilized. As a carbon energy source, dried orange peels extracts were added at a final concentration of 2%. The pH was adjusted to 6.8 using 2 M NaOH. Batch fermentation was carried out in duplicate and under continuous stirring at 100 rpm using a magnetic stirrer. Fermentation was allowed for 72 h at 35°C and samples from the medium were withdrawn periodically from the replicated fermented flasks to determine bacterial cell growth, ethanol productivity, final pH value and residual sugar content.

Analysis

Optical density (OD) was measured at 600 nm to estimate the cell growth. The pH of the supernatant was measured with a pH meter. Ethanol was analyzed using a gas chromatography equipped with a flame ionization detector and a BP21 capillary column (25-m length x 0.53-mm internal diameter x 0.5-µm film thickness). The temperature of the injector and detector were set at 150 and 200°C, respectively. The oven temperature was initially maintained at 40°C for 1 min and then increased to 130°C at a gradient of 20°C per minute. Helium was used as carrier gas with 1-propanol as the internal standard [14]. All tests were run in duplicate with three repetitions, and results were expressed as the average of all the repetitions. By using glucose as a standard, total sugars were analyzed by the method proposed by Dubois et al.[15], which is based on the phenol sulfuric acid reaction. Using D-galacturonic acid as a standard sugar, the reducing sugar was determined by 3, 5- dinitrosalicylic acid (DNS) method [16].

Optimization of process parameters

The various process parameters were optimized for maximal ethanol production were orange peels concentration, carbon source, nitrogen source, inoculum concentration, pH, temperature and incubation time.

A set of flasks with different substrate concentrations ranged from 1-10 % were inoculated and incubated at 35°C for 72 h. After incubation, samples were withdrawn and tested for all parameters described in this work. To find a suitable additional carbon source for bio-ethanol production by *Bacillus subtilus*, carbon sources; glucose, galactose, sucrose, maltose, xylose, raffinose, arabinose, cellulose, lactose, starch, fructose, and molasses were added at 1% to minimal medium, fortified with orange peels. The effect of sucrose and lactose at concentrations of 0.5, 1, 1.5, 2, 2.5 and 3% were studied on ethanol production. Yeast extract, peptone, malt extract, beef extract, urea, ammonium sulphate, ammonium nitrate, ammonium chloride, sodium nitrate, potassium nitrate, and asparagin, were selected as nitrogen sources for optimization process. The nitrogen sources were used at 2 g/L in basal minimal medium. The effect of ammonium chloride and corn steep liquor at concentrations of 1, 2, 3, 4, 5 and 6 g/L were studied on bio-ethanol production.

A set of flasks containing minimal media with different volumes of inoculum (approximately 108 CFU/ml), viz. 2, 4, 6, 8, 10 and 12% (v/v) were studied for the effect of inoculum concentrations. The flasks were incubated at 35°C for 72 h. The pH of the production medium was adjusted to 5.0, 6.0, 7.0, 8.0 and 9.0 with 1N NaOH and 1N HCl. The bio-ethanol production was carried out at 35°C to study their effect. The fermentation was carried out at different temperatures such as 20, 25, 30, 35 and 40°C to study their effect on ethanol production. The culture filtrates were then collected and assayed. Different incubation times (24, 48, 72, 96 and 120 h) were employed to study their effect on bio-ethanol production. The culture filtrates were collected at respective time intervals (24 h) and assayed.

3. Results and Discussion

Growth and production of bio-ethanol from fermentable sugars in orange peels is an alternative to utilize industrial citrus processing waste and avoid disposal-associated problems. The removal of the limonene can be done in many ways. In a heat treatment method, the orange peels are treated at 150°C (70 psi) by injecting high pressure steam and bioconversion of orange peels, hydrolysis of polymers is essential. Hydrolysis can be carried out either chemically, where acid hydrolysis dominates, or enzymatically. Enzymatic hydrolysis is an efficient method to release almost all carbohydrates present in the orange peels which can further be fermented to bio-ethanol. The development of a cost-effective method in which a high proportion of carbohydrates could be released will help to commercialize the processes using orange peels as raw materials.

Dilute-acid hydrolysis is a fast and economically feasible approach that is widely used and application of high temperatures in this method accelerates the rate of sugar decomposition. Various process parameters influencing fermentation rate and bio-ethanol production were optimized. The strategy followed was to optimize each parameter, independent of the others and subsequently optimal conditions were employed in all experiments.

Nutritional requirements

Nutrient sources were found to be one of the important factors for bio-ethanol production. On studying the ability of *Bacillus subtillis* to utilize hydrolyzed orange peel wastes with no need of supplying any additional nutrient to produce bio-ethanol, it was found that a concentration of 8% (w/v) optimum for bio-ethanol production was reached at 18.90 by *Bacillus subtillis* (Table 1). Beyond 8%, the substrate concentrations decreased the bio-ethanol production.

Table 1: Influence of additional carbon sources (1%) on growth and production of bio-ethanol by Bacillus subtillis cultured in minimal salt medium containing 8% orange peels

	Bacillus subtillis				
Carbon source	Ethanol yield (g/L)	Total sugar(g/L)	Reducing sugar(g/L)	Biomass (OD at 600nm)	pH
Glucose	47.00	83.28	46.96	1.210	5.00
Galactose	43.80	72.56	43.72	1.445	6.00
Sucrose	56.50	70.90	11.86	1.585	5.00
Maltose	46.80	75.82	82.76	1.133	5.90
Xylose	50.00	87.00	18.90	1.601	4.90
Cellulose	15.50	76.12	13.42	2.493	6.60
Lactose	47.10	77.54	48.56	1.232	6.50
Starch	40.30	72.54	34.54	2.223	6.00
Fructose	43.40	75.00	48.88	1.304	5.40
Molasses	21.20	79.36	15.30	1.716	6.10

It is evident from results represented in Table 1 that by increasing bio-ethanol production, the pH of the culture decreased. The reduction in pH provides a convenient marker for the completion of fermentation and appears to coincide with maximum ethanol concentration. Since carbon is considered as the primary nutrient for the bacteria, further experiments were done to explore the enrichment of carbon sources with the objective of maximizing bio-ethanol production. The influence of additional carbon sources on bio-ethanol production was studied by adding various carbon sources like glucose, galactose, sucrose, maltose, cellulose, lactose, starch, fructose, and molasses to the culture medium. Enhanced bio-ethanol production (56.50 g/l) by *Bacillus subtilis* was found in medium amended with sucrose. These results mean that the bio-ethanol produced in this study was higher than that produced by *Kluyveromycesmarxianus* (37.1 g/l) and *Saccharomyces cerevisiae* (40.9 g/l) grown on hydrolyzed orange peel waste [17]. Sugar concentration is also critical in fermentation process and influencing the rate of bio-ethanol production. Initial sugar concentration has also been found to determine the amount of alcohol [18].

Table 2: Effect of different nitrogen sources on growth and production of bio-ethanol by Bacillus subtillis

	Bacillus subtillis				
Nitrogen source	Ethanol yield (g/L)	Total sugar (g/L)	Reducing sugar (g/L)	Biomass (OD at 600nm)	pH
Yeast extract	61.70	62.85	37.26	2.297	6.50
Peptone	51.10	89.81	47.94	2.356	6.80
Malt extract	58.80	60.27	30.56	1.540	6.20
Urea	55.00	54.51	29.26	1.801	7.40
Ammonium sulphate	59.20	60.22	39.80	1.919	5.40
Ammonium nitrate	59.00	84.76	44.16	1.562	6.30
Ammonium chloride	67.30	98.80	35.70	1.894	5.00
Sodium nitrate	36.70	63.93	34.82	2.343	7.30
Potassium nitrate	42.70	88.70	9.70	2.249	7.30
Asparagin	52.10	94.45	53.24	2.602	7.70

Orange peels contain different carbohydrate polymers which makes it attractive as a raw material for production of metabolites such as ethanol by suitable microorganisms. The total sugar content of orange peel varies between 29 and 44%, soluble and insoluble carbohydrates being the most abundant and economically interesting constituents of this residue. Approximately 50% of the dry weight of orange is soluble in alcohol, and soluble sugars are the major components also of this fraction. Glucose, fructose and sucrose are the main sugars, although xylose can also be found in small quantities in orange peel. Insoluble polysaccharides in orange peel are composed of pectin, cellulose and hemicelluloses. Pectin and hemicelluloses are rich in galacturonic acid, arabinose and galactose. Glucose is the dominant sugar in the cellulosic fraction, which also contains some quantities of xylose and arabinose, traces of galactose and uronic acids, and in some instances mannose. On the other hand, lignin seems to be absent in these tissues. Next to carbon, nitrogen served as important nutrient source for bio-ethanol production as it has a very important role in microbial growth and enzymes production.

Hence, different nitrogen sources like peptone, malt extract, casein, protease peptone, urea, ammonium sulphate, ammonium nitrate, ammonium chloride, sodium nitrate, potassium nitrate, and asparagin were applied as nitrogen sources for bio-ethanol production. Ammonium chloride was found to be the best nitrogen source as it increases bio-ethanol production up to 67.30 g/l by *Bacillus subtilis*.

Environmental factors

In the conversion process of sugar to ethanol, growth of microorganisms was highly linked with stress or environmental factors in the culture medium, which of these factors is essential to achieve a successful fermentation and an increased ethanol yield. The effect of different inoculum size, including 2, 4, 6, 8, 10 and 12% (v/v), on bio-ethanol production were studied. In a medium optimized for carbon and nitrogen sources, the inoculum size resulted in maximum ethanol yield 90.20 for *Bacillus subtilis* was 10 % (v/v). An increase in inoculum level is well known to reduce the lag phase. As obvious in Table 3, the biomass increased steadily when the inoculums varied from 6 to 10%. There was a notable decrease when the inoculums varied from 10 to 12% in case of *Bacillus subtilis*. The results presented in Table 4 demonstrated that pH 7.0 and 8.0 favored ethanol production by *Bacillus subtilis* the maximum of 92.25.

Table 3: Effect of inoculums size on growth and production of bio-ethanol by Bacillus subtillis

	Bacillus subtillis				
Inoculum size (%)	Ethanol yield (g/L)	Total sugar g/L)	Reducing sugar(g/L)	Biomass (OD at 600nm)	pH
2	57.70	63.78	38.46	1.942	6.50
4	76.30	51.50	27.18	1.986	6.30
6	79.00	46.80	24.06	2.011	6.00
8	85.50	44.95	21.18	2.330	6.00
10	90.20	42.40	19.90	2.540	5.50
12	82.76	45.48	25.23	1.116	5.90

Table 4: Effect of initial pH of media on growth and production of bio-ethanol by Bacillus subtillis

	Bacillus subtillis				
Inoculum size(%)	Ethanol yield (g/L)	Total sugar(g/L)	Reducing sugar(g/L)	Biomass (OD at 600nm)	pH
5	48.50	79.02	43.92	1.189	3.00
6	60.20	54.88	29.50	1.457	3.00
7	92.25	43.00	19.68	2.540	5.80
8	70.20	46.96	22.44	1.940	6.50
9	66.70	50.00	26.20	1.720	7.00

Growth temperature is another critical parameter that needs to be controlled. For the temperatures tested, *Bacillus subtilis* showed maximum ethanol productivity (92.25) at 35°C. It has been observed that in both lower and higher temperatures, the ethanol production was sharply decreased.

4. Conclusion

The period of fermentation depends upon the nature of medium, fermenting organisms, concentration of nutrients and the process physiological conditions. The effect of incubation period on bio-ethanol production was tested in this work. . From the studies, it was found that the maximum amounts of ethanol produced using orange peels at concentration of 8%. 67.20 g/L bio-ethanol was produced with the addition of 2% sucrose by *Bacillus subtilis.* The maximum amount of bio-ethanol was produced as 75.20g/L using *Bacillus subtilis* by adding ammonium chloride to the medium at 4.0g/L. The influenced process parameters such as inoculum concentration, pH, temperature and incubation time were found to be optimum at 10%, 7, 35°C, 72 h respectively. In the presence of *Bacillus subtilis* the bio-ethanol production was found to be 92.25% at these optimum conditions.

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6. Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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