Use of Grass Sap as an Ingredient in Lysine Production

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Abstract

The objective of this research was to produce cheap lysine by using grass sap as an ingredient. Four different formulas of fermentation media were prepared, namely media A, A1, B, and C, respectively. Three different strains of microorganisms, IFA 129 (*Brevibacterium* E 531), 133 (*Corynebacterium* glutamicum), and 134 (*Brevibacterium* lactofermentum) were used as lysine producers. The experiment was run under a temperature of 30°C and at a pH ranging from 7.2 to 7.5. IFA 129 and 133 were proven to be the best potential strains for producing a high yield of lysine in all media. Medium C (mainly grass sap mixed with molasses) was found to be the suitable formula for lysine production, and was an especially good source of growth nutrient and carbohydrates. The highest amount of lysine produced after 72 hours of Medium C were 32.65 (IFA 129), 40.32 (IFA 133), and 14.21 (IFA 134) g/l, respectively. Media B, A1, and A were the second, third and fourth highest amount of lysine produced, respectively. In addition, corn steep liquor was proven to be a good substitute for yeast extract, a source of growth nutrient especially for methionine and threonine. However, further work using produced lysine in animals be conducted.

Keywords: Grass-sap; IFA strains; Fermentation; Lysine

1. Introduction

Lysine is considered to be essential for all aspects of animal production, however, animals cannot synthesize it. Several advantages of amino acid supplementation (such as lysine) include increased growth performance, improved animal health, and better meat quality due to a reduction in fat. In addition, by adding the limiting essential amino acids to rations, nutritional requirements are satisfied and protein levels reduced. This can lead to a decrease of 20 to 30 % in nitrogen excretional pollution [3]. Therefore, lysine is widely used as a feed additive. The decreasing area of arable land which is used for crop production and the increasing population lead to an increased use of crop products mainly for human instead of for animals. Because of this, there are attractions in considering food by-product materials in animal feed as they help to avoid the immediate competition for food resources between humans and animals. Furthermore, the use of food by-products as raw materials in the process of producing lysine will lead to a decrease of animal feed cost which is the largest single item of expense in the production of milk, meat and eggs, often accounting for up to 70 % of the total costs [7, 10].

An increase in the use of food by-products is necessary because several by-products produced in large amounts in the food industry may cause pollution unless they are used as feedstuff. In addition, the pollution control regulations, which were relatively loose until a few decades ago, are now extremely tight [2].

One of the methods of upgrading wastes and by-products is fermentation. There has been considerable interest in the use of microorganisms to upgrade waste and by-products and to provide a source of good quality protein for the animal feed industry. Many types

of microorganisms, including bacteria, fungi and algae, have been investigated. They are a particularly useful means of detoxifying troublesome pollutants (e.g. liquid discharges from food processing or blood from slaughterhouses) of high biological oxygen demand and having a high content of suspended solids, and of concentrating protein. In addition, inorganic substrates may be used (such as effluent from pulp mills). In fact the number of substrates capable of being fermented by, for example, yeast, is considerable. A consequence of this is that the nutritive value of the final product may be variable, a point that is also relevant when considering both the type of microorganism used and harvesting conditions [11].

L-lysine is produced in improved yield by culturing microorganisms of the genus *Corynebacterium* and *Brevibacterium* having the ability to produce L-lysine in a culture medium, the microorganisms being characterized by either a resistance to two or more antibiotics or resistance to one or more purine analogs and/or to one or more pyrimidine analogs [5]. L-lysine is accumulated by L-alanine, an L-lysine producing mutant that belongs to one of the genera *Brevibacterium*, *Corynebacterium* and *Arthrobacter* [4].

The fermentation using cane blackstrap molasses in a 2 kl fermentor utilized the following media. The first seed medium contained 2% glucose, 1% peptone, 0.5% meat extract and 0.25% NaCl in tap water. The second seed medium consisted of 5% cane blackstrap molasses, 2% $(NH_4)_2SO_4$, 5% corn steep liquor and 1% $CaCO_3$ in tap water and the fermentation medium itself contained 20% cane blackstrap molasses and 1.8% soybean hydrolyzate in tap water. The fermentation was carried out at 28 degrees Celsius using *Cory.glutamicum*. The amount of lysine accumulated after 60 h is 44 g / l [6]. In addition, the effective biosynthesis of lysine (up to 29 g/l in 48 hr) was noted during cultivating of the auxotrophic strain *Brevibacterium sp.531* in fruit and vegetable mediums using Chinese cabbage juice [8].

AGROFERM [1] stated that plant juice is converted to a nutrient for bacteria that demand vitamins and amino acids and that form organic acid or amino acids. The process is comprised of: (1) heat treating between 24 hr at 55 deg and 10 min at 120 deg; (2) cooling to 50-60 deg; (3) adjusting with base, preferably ammonia, to pH 7.5-8.5; and (4) hydrolyzing by proteolytic enzymes, especially protease or peptidase with continuous agitation and addition of base to maintain the pH. The enzymes are those that occur naturally in grass juice, for example, or formed by microorganisms that are cultivated in the plant juice or in a different nutrient. The proteolytic enzyme is alcalase. The process allows cheap and simple conversion of plant juice, especially grass-sap, to organic (amino acids), especially glutamic acid, L-lysine and threonine. In an example, potato juice, a starch production waste, was hydrolysed using 0.1% alcalase solution. Growth of *Corynebacterium glutamicum* was 6.6 U (c.f. 0.5 on untreated potato juice), *Brevebacterium lactofermentum* (ATCC 21798) was 4.4 (c.f. 1.2) and ATCC 13869 was 6.5 (c.f. 1.8). The objective of this research was to produce cheap lysine by using grass sap as an ingredient.

2.Materials and methods

Cultures of IFA 129, 133, and 134 (homoserine auxotroph) were used as lysine producers. They were cultivated in 500 ml Erlenmeyer flasks, containing 20 ml of nutrient media, on a shaker for 24 hours.

Grass harvested from a surrounding area of IFA-TULLN was mixed with water in a blender. This mixture was homogenized and was then extracted into a grass-sap. Next, it was mixed with synthetic glucose in the amount of 30 g/l (medium A) and 50 g/l (medium B), and with beet molasses equal to the amount of total carbohydrate 100 g/l (medium C). Corn steep liquor was also used as a substitute for yeast extract (Medium A.1). The following substances were added into different fermentation media (in g/l): $(NH_4)_2SO_4$ 30 ; $CaCO_3$ 30 ; and biotin 0.0003. The sterile fermentation medium was inoculated with seed (IFA 129, 133, 134) in the amount of 10%.

The batch fermentation was conducted at 30 degrees Celsius and at a pH 7.2-7.5 in 500 ml shaker flasks at a speed of 220 rpm. Samples were taken every 24 hours ,until the last day, which was at 72 hours for pH, lysine and total reducing sugars determinations.

3.Results and discussion

The basic characteristics of grass-sap, molasses and corn steep liquor were analyzed.NH^{4+} and crude protein were determined by the Kjeldahl method. The sugar and amino acids contents were analyzed by HPLC. Table 1 shows the results of these analyses.

Table 1: The characteristics of grass-sap, molasses and corn steep liquor

Parameter	Raw material		
	grass-sap	molasses	corn-steep liquor
NH_4^+ (g/l)	0.70	0.70	5.21
Crude protein (g/l)	3.21	116.59	178.38
Total carbohydrate (g/l)	7.53	641.30	3.32 (glucose)
Total amino acids	16.11 mg/g	35.84 mg/g	N.D.
Methionine	0.008 mg/g	0.25 mg/g	6.45 g/l
Threonine	0.24 mg/g	1.29 mg/g	7.02 g/l

N.D. = not determined

The amounts of lysine produced on different media after 72 hours are shown in Table 2 and Figure 1. The lysine produced every 24 hours by Media A, A1, B, and C are shown in Figure 3,4,5 and 6, respectively.

The results of total reducing sugars of medium C are presented separately in Figure 2. Table 3 represents the sugar consumption in Medium C during fermentation by different strains of microorganisms.

Table 2: The amount of lysine produced on different media after 72 hours

Medium	Lysine (g/l)	YP/S (%)	Pr (g/l.hr)
(A) Grass-sap + synthetic glucose (30 g/l)+yeast extract (20 g/l):			
IFA 129	2.44	8.13	0.034
IFA 133	10.66	35.53	0.148
IFA 134	3.08	10.27	0.043
(A1) Grass-sap + synthetic glucose (30 g/l)+corn steep liquor (50 g/l):			
IFA 129	10.35	34.5	0.144
IFA 133	13.14	43.80	0.182
IFA 134	4.85	16.17	0.067
(B) Grass-sap + synthetic glucose (50 g/l)+corn steep liquor (50 g/l):			
IFA 129	23.68	47.36	0.329
IFA 133	21.79	43.58	0.303
IFA 134	2.99	5.98	0.042
(C) Grass-sap + molasses (100 g/l)+yeast extract (20 g/l):			
IFA 129	32.65	32.65	0.453
IFA 133	40.32	40.32	0.560
IFA 134	14.21	14.21	0.197

YP/S = Yield of Lysine (%), Pr = Productivity of lysine (g/l hr)

Table 3: Sugar consumption of medium C

Medium C	Original sugars (g/l)	Final sugars (g/l)	Consumption (%)
IFA 129	100	11.95	88.05
IFA 133	100	5.09	94.91
IFA 134	100	8.22	91.78

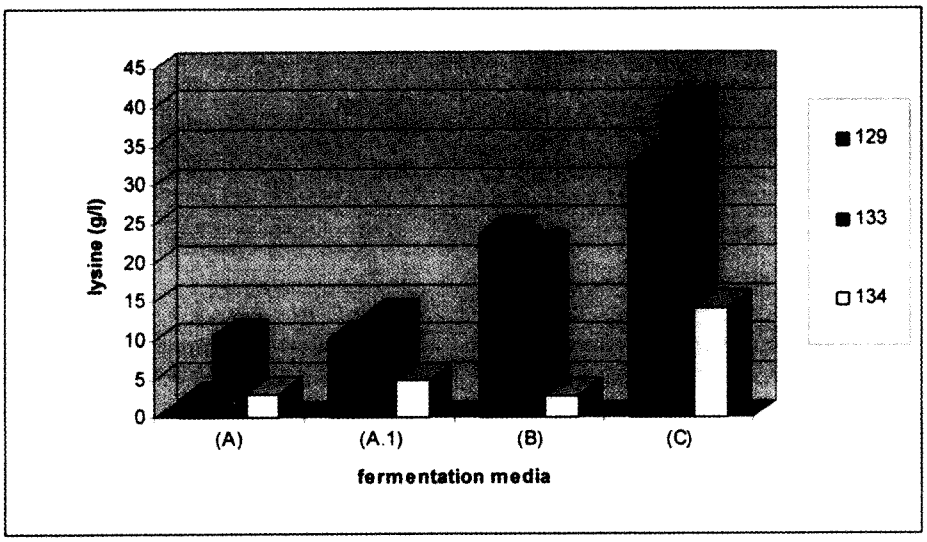

Figure 1: Lysine concentration (g/l) of different media after 72 hours

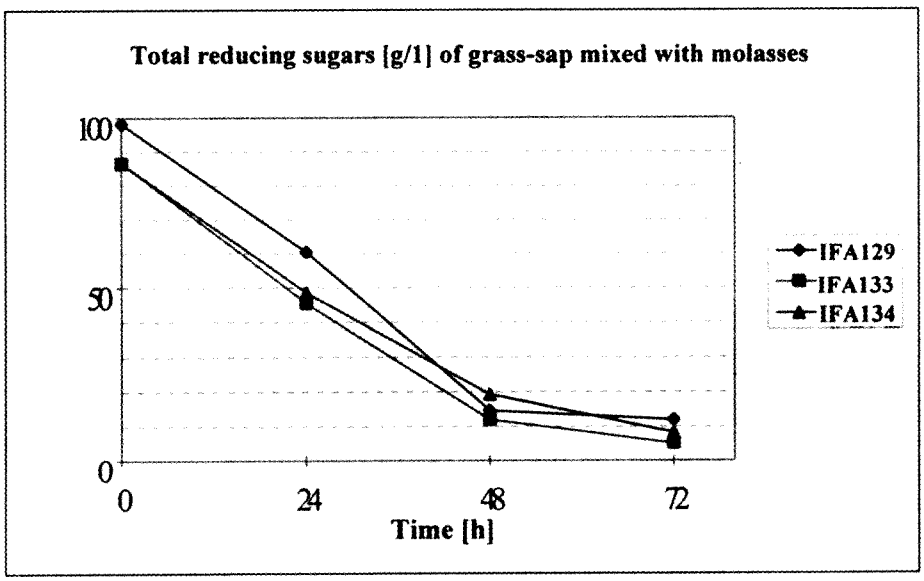

Figure 2: Total reducing sugars (g/l) of Medium C

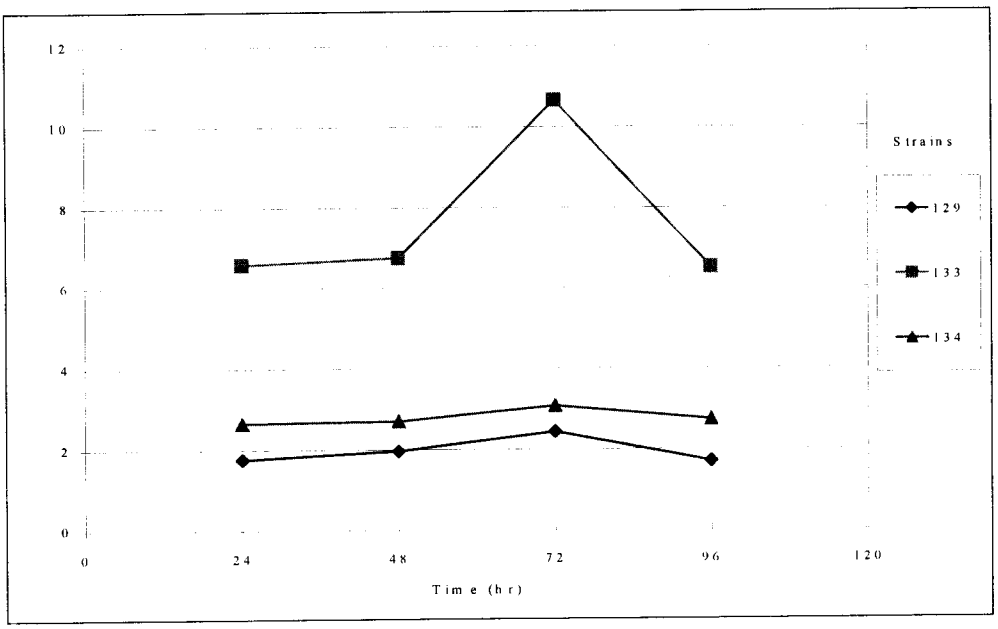

Figure 3: Lysine production of different bacterial strains on Medium A.

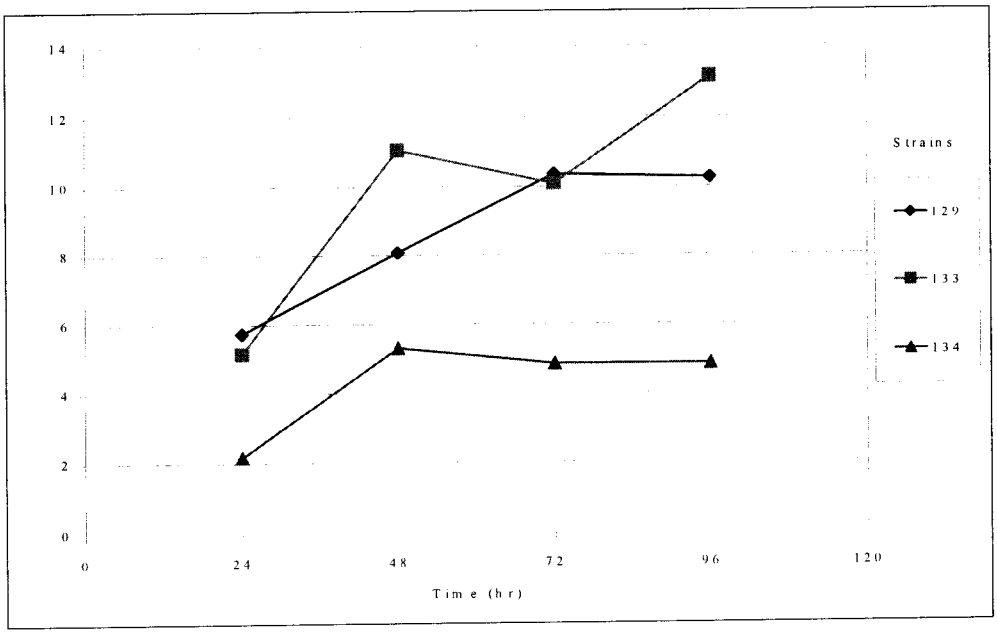

Figure 4: Lysine production of different bacterial strains on Medium A1.

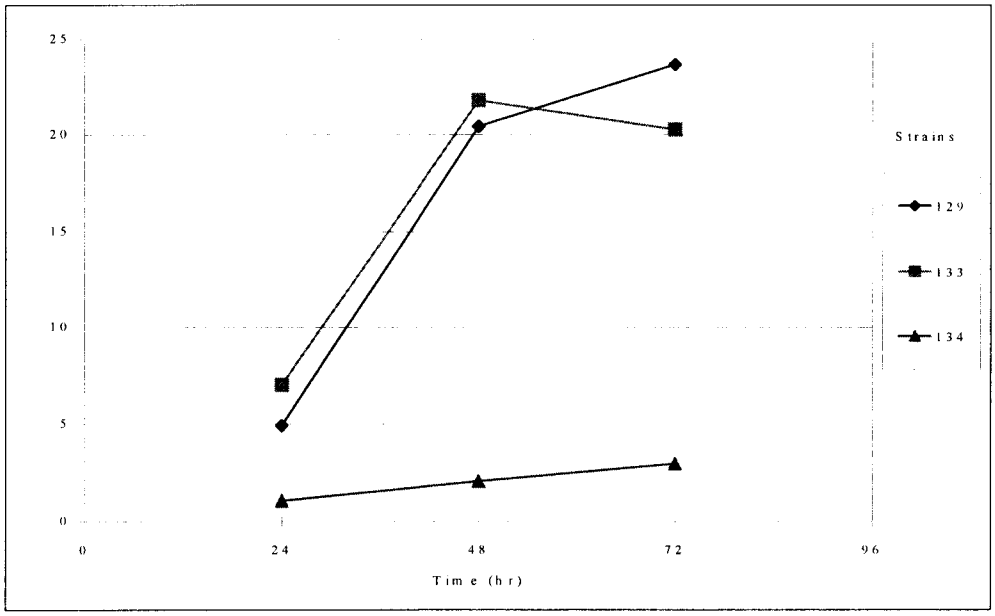

Figure 5: Lysine production of different bacterial strains on Medium B

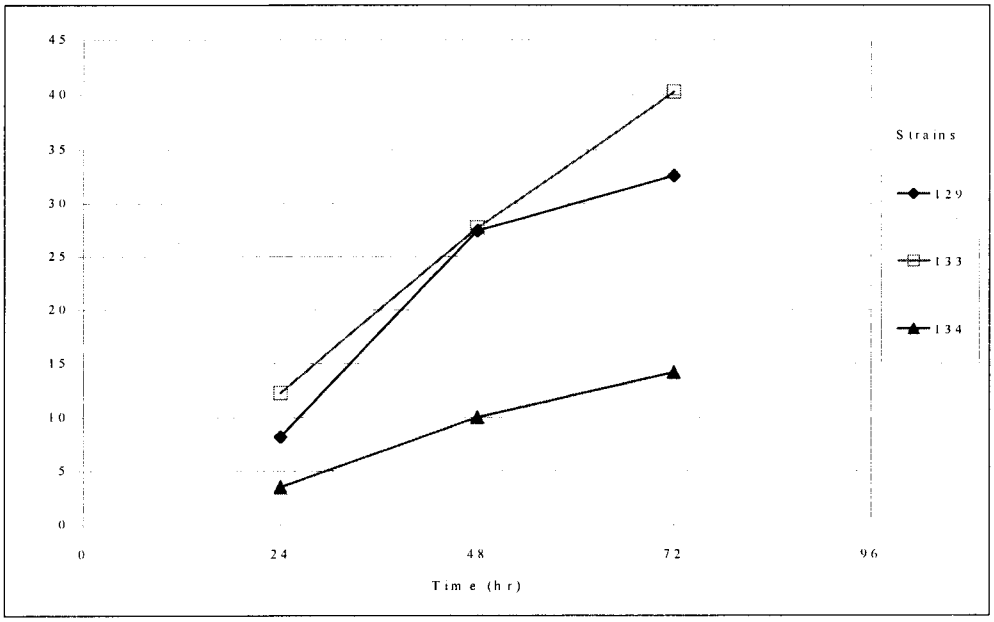

Figure 6: Lysine production of different bacterial strains on medium C

Grass-sap was composed of a total carbohydrate of 7.53 g/l with amounts of NH_4^+ and crude protein measuring 0.70 g/l and 3.21 g/l, respectively. It contained L-methionine and L-threonine in the amount of 0.008 mg/g and 0.24 mg/g with total amino acid of 16.11 mg/g. Its' pH was 6.97. According to AGROFERM [1], plant juice is converted into a nutrient for bacteria that demand vitamins and amino acids and that form organic acid or amino acids.

Medium A was prepared with the addition of ammonium sulfate, yeast extract, amino acids, and other inorganic substances. The highest amount of lysine produced was 10.66 g/l by IFA 133 after 72 hours of fermentation followed by IFA 134 and 129 at 3.08 and 2.44 g/l, respectively (Table 2 and Figure 1,3). These results clearly support an accumulation of lysine with a rising trend of pH ranging from 7.24 to 8.04.

The substitution of corn steep liquor for yeast extract and other growth nutrients was conducted in Medium A1. The lysine production improved significantly when compared to the results obtained in Medium A with the use of all three strains (IFA 129, 133, and 134). The largest increase was 13.14 g/l by IFA 133 after 96 hours of fermentation (Table 2 and Figure 1,4). Additionally, IFA 133 also resulted in the highest lysine yield and productivity (Table 2). The pH data gave a clear indication of lysine accumulation with a rising trend ranging from 6.5 to 8.76. The longer fermentation period needed to obtain the highest yield of lysine production (96 hours of fermentation period) is consistent with the results of fermentation using corn steep liquor as a source of growth nutrient. This clearly indicates that corn steep liquor is a very good source of protein (crude protein = 178.38 g/l). In addition, this corn steep liquor also contained a mixture of different amino acids (especially L-threonine 7.02 g/l and L-methionine 6.46 g/l), which could be used as a source of growth nutrients for L-lysine producing bacteria.

The composition of Medium B was based on Medium A1 but with an increased amount of added glucose from 30 g/l to 50 g/l. The amounts of lysine produced by IFA 129 and 133 were sharply improved to 23.68 g/l and 21.79 g/l, respectively (Table 2 and Figure 1,5). This clearly indicates the limitation of a carbon source in Medium A1. When considering the pH data, it clearly shows the accumulation of lysine towards the end of fermentation with a considerable increase of pH after 24 hours in the range 5.79 to 8.76.

The results obtained from Medium B suggest the use of molasses as a carbon source conducted in Medium C, with the use of yeast extract instead of corn steel liquor and without adding other growth nutrients. The reason for not using corn steep liquor and molasses together comes from the results obtained in Molasses 2 (with corn steep liquor) and Molasses 3 (with yeast extract). The amount of added molasses was equivalent to 100 g of glucose/l. The amounts of lysine produced were significantly increased in all three strains up to the highest of 40.32 g/l (by IFA 133), 32.65 g/l (by IFA 129), and 14.21 g/l (by IFA 134) (Table 2 and Figure 1,6). The increasing pH (in the range 6.67 to 8.31) and the decreasing total reducing sugars (ranging from 98.22 to 5.09 g/l) strongly supports the production of lysine towards the end of fermentation. This outcome shows that carbohydrates are the main source of the energy needed for the vital activity of microorganisms ,and that molasses can be used as this source. The reason for this conclusion is that molasses was a good source of carbohydrate and protein with contents of 641.30 g/l and 116.59 g/l respectively. In addition, it also contained total amino acid of 35.84 mg/g, including 0.25 mg/g methionine and 1.29 mg/g threonine (Table 1).

Additionally, it also indicates the importance of grass-sap as a source of amino acids (at the total amount of 16.11 mg/g), especially L-threonine (at 0.24 mg/g) and L-methionine (at 0.008 mg/g) (Table 1).

The media formula of Medium C with the use of molasses mixed with grass-sap and the use of yeast extract instead of corn steep liquor is reminiscent of the work done by PHAM [9]. The mixing of sugar cane juice enriched with coconut water was used as a raw material in lysine production with a homoserine auxotroph 9NG7 as lysine producing bacterium. The lysine yield increased 1.5 fold to 16.9 g/l when sugarcane with coconut water (66 g/l total sugars) was used as the culture medium and corn steep liquor was omitted.

4.Conclusion

In conclusion, Medium C, with the highest amount of lysine yield at 40.32, and 32.65 g/l by IFA 133, and 129, respectively should be used in further tests in a pilot scale in order to be able to produce it commercially.

Grass-sap mixed with molasses (Medium C) was proven as a good fermentation medium composition, especially as sources of growth nutrients and carbohydrate in producing cheap lysine. The yield of lysine mainly depends on the amount of carbohydrate and the favorable ratio of growth substances in the fermentation media. Corn steep liquor has been proven to be a good substitute for yeast extract, a source of growth nutrients.

Further experiments should be conducted with the use of lysine produced from Medium C in order to check its quantity and quality as feed supplements.

5. Acknowledgments

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