Sugar Substrates for L-Lysine Fermentation by Ustilago maydis

A. Sánchez-Marroquín, M. Ledezma, and R. Carreño

Facultad de Ciencias, Universidad Central de Venezuela, Apartado 5097, Caracas 104, Venezuela

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The extracellular production of L-lysine in media with cane sugar, blackstrap molasses, or clarified sugar-cane juice by a previously obtained mutant of Ustilago maydis was studied. Enzymatically inverted clarified juice (medium J-3) gave 2.9 g of lysine per liter under the following conditions: inoculum, 5%; pH 5.8; temperature, 30 °C; KLa in the fermentors, 0.41 mmoles of O2 per liter per min; fermentation time, 72 hr. The concentrate, obtained by direct evaporation and drying of the fermentation broth, could be used as a possible feed supplement because of its amino-acid and vitamin content.

Five auxotrophic mutants of Ustilago maydis, obtained by a combination of ultraviolet and ethylenemine treatment, were previously studied (8) on more than 60 media with agave juice (aguamiel), corn-stEEP liquor, corn oil, and ammonium salts as the main constituents. Yields as high as 2.5 g per liter were reached in 300-liter fermentors by using mutant UV-ET-15.

Since this is a high yield for this particular species, it was decided to study three other inexpensive substrates: commercial sucrose, sugar cane clarified juice ("clarified juice," International Society of Sugar Cane Technologists terminology), and blackstrap molasses and to continue working on the development of new high-yielding mutants. Among these, homoserine-γ, methionine-γ, and threonine-requiring mutants were found capable of producing acceptable amounts of L-lysine.

The present report deals with studies carried out to gain maximum yields of free L-lysine by using such mutants. However, the yields here presented are far below any value that would warrant production of this amino acid by U. maydis on an industrial scale. Bacteria will probably continue to be the selected microorganisms for this purpose at the present time, but the possibility exists that by improving the yields of U. maydis through induced genetic changes in the strains, by selection of fermentation media, and by adequate bioengineering operations fungi may be used for the commercial production of L-lysine.

MATERIALS AND METHODS

Cultures. Mutants of U. maydis were kept in potato-dextrose-agar slants or on sterilized corn, and monthly transfers were made. Inoculum was prepared from 4-day cultures at 28 °C as follows. A loopful was transferred to tubes containing 10 ml of a sucrose medium, composed of (per 100 ml) sucrose, 0.25 g; peptone, 1.0 g; NaCl, 0.5 g. After 38 hr of cultivation in a rotary shaker, 5% inoculum was used to seed Dulany's medium (3) in Erlenmeyer flasks. After 48 to 72 hr of agitation at 250 rev/min and 28 °C, sufficient seed was taken to inoculate the fermentation media at a level of 5%.

Media. To compare the activity of the strains, Dulany's glucose medium (3), B glucose medium (8), and agave juice media (26, 48, and 19 (8)] were used. The effect of ammonia nitrogen and naturally occurring nitrogen sources on L-lysine formation by mutant UV-ET-15 was studied in Dulany's glucose medium with 1% corn oil added.

The effect of various commercial carbon sources was studied in medium 26-b, which was composed of ammonium phosphate, 0.8 g; K2HPO4, 0.1 g; ammonium acetate, 0.1 g; corn steep liquor (50%), 2.0 g; corn oil, 1.0 ml; CaCO3, 0.17 g; sugar solution, 100 ml (5% sucrose basis).

Fermentations were carried out in this medium with the following carbon sources: medium S-1 (commercial sucrose as received); S-2 (invertase-treated sucrose); M-1 (blackstrap molasses as received); M-2 (blackstrap molasses treated with 10 mg per liter of K4Fe(CN)6 at 80 °C during 6 hr, centrifuged, and filtered); M-3 (ferrocyanide-treated molasses, hydrolyzed with invertase); J-1 (clarified juice, as received); J-2 (clarified juice treated with ferrocyanide as indicated before), and J-3 (clarified juice, ferrocyanide-treated and invertase-treated). Commercial invertase (0.1%) was used for the treatment of these sugar substrates at 50 °C for 48 hr. When CaCO3 was used, it was sterilized separately and then incorporated in the medium. Media were autoclaved at 115 °C for 20 min, cooled, and then inoculated with 5% seed.

Conditions. The inoculated Erlenmeyer flasks were mechanically agitated for 120 hr at 250 rev/min in a
New Brunswick rotary shaker. The incubation temperature was 30° C; pH was adjusted at 5.8 to 6.2 in all fermentation media. The same pH and temperature conditions were established for the 7.5-, 20-, and 100-liter fermentors. The 7.5-liter fermentors corresponded to model F-7 of New Brunswick Scientific Co., equipped with pH, temperature, and foam automatic controls, one disc sparger, two turbine impellers, and four baffles. The 20-liter fermentor was a French E.I.V.S.-672 glass model with three flat-blade impellers, one disc sparger, and no baffles. The 100-liter fermentor was included in a steel pilot plant designed by Olsa from Milan, Italy; this fermentor has one turbine impeller, a ring sparger, and four baffles. Aeration, agitation, and other operational conditions are indicated in the respective tables.

Concentrate. A lysine concentrate was obtained by evaporation and spray-drying of the fermentation broth from the 100-liter fermentor, with medium J-3 as a substrate. The material was then passed through an electrical mill, and the brown powder obtained was submitted to chemical analysis.

Determinations. Reducing sugar, pH, viscosity, oxygen-transfer rate, and mycelial weight were determined as indicated previously (8). The microbiological standard method with *Pediococcus cerevisiae* P-60, occasionally checked against paper chromatographic techniques, was used in the estimation of L-lysine. For the chemical determinations of the amino acid concentrate, previous hydrolysis with 6 N HCl for 22 hr at 100° C was made, and then AOAC analytical methods were followed (1). The amino acid pattern was detected by means of a Beckman model B automatic analyzer. For the digestibility tests of the powdered concentrate, defatting was performed in a Goldfish apparatus; the AOAC method (1) was followed by using a buffer boric acid solution in place of the conventional acid solution to receive the distilled ammonia.

**RESULTS**

Table 1 shows the L-lysine yields in five media by the IFSC 65-1 parent strain of *Ustilago maydis* and the four mutants obtained from it. The UV-ET-15 homoserine-requiring mutant had been previously studied (8) in the agave juice media 26-a, 48, and 19. Results indicate that this mutant gave the highest yields in most of the media tested, and it was therefore selected for all subsequent experiments.

The effect of three ammonium and two naturally occurring nitrogen sources on L-lysine formation by this particular strain on Dulaney's glucose medium with 1% corn oil is shown in Table 2. The three ammonium salts at their respective optimal concentrations gave almost identical results, and their lysine yields were higher than those obtained with corn steep liquor or yeast extract. Yeast extract appears to affect both growth and lysine synthesis, whereas corn steep liquor and yeast extract apparently are used for growth only.

Some other naturally occurring nitrogen sources such as gelatin, peptone, corn meal, cotton seed meal, and soya flour did not consistently improve yields. The addition of some probable precursors such as aminoacidic and ketoacidic acids did not influence results in any way.

When the effect of some commercial carbon sources was studied, the addition of corn steep liquor seemed favorable for good and reproducible lysine production. Results in shaker-flask experiments shown in Table 3 refer to medium 26-a inoculated with the UV-ET-15 mutant. Clarified juice appeared to be a better substrate than sucrose or blackstrap molasses. A slight increase in lysine yields or a shortening of the fermentation time were apparent when these substrates were previously hydrolyzed by means of commercial invertase, especially in the case of clarified juice. Growth and sugar consumption were affected in a similar manner. The best yields were approximately 2 g of lysine per liter. Ferrocyanide treatment was of no particular benefit.

Fermentations carried out in 7.5-liter fermentors by using five selected commercial sugar media usually gave inconsistent results when no corn steep liquor was added to the media or when the aeration and agitation conditions were not

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**Table 1. L-lysine formation (mg/ml) in glucose media by parent strain and five mutants of Ustilago maydis**

| Strains            | Dulaney's medium | Other glucose media |
|--------------------|------------------|---------------------|
|                    | 26-a  | 48    | B     | 19    |
| 65-1 IFSC (parent) | 0.17  | 1.63  | 1.17  | 1.59  | 1.46  |
| UV-ET-15 (homoserine) | 1.66  | 2.67  | 1.44  | 2.76  | 2.18  |
| UV-78 (threonine)  | 1.16  | 2.11  | 1.98  | 2.15  | 1.90  |
| UV-ET-89 (threonine) | 1.77  | 2.48  | 2.10  | 1.99  | 1.75  |
| UV-15-M (methionine) | 1.98  | 1.96  | 2.12  | 2.18  | 2.05  |

* A 50-ml amount of medium was placed in 250-ml Erlenmeyer flasks. Inoculum consisted of 5% of a 48- to 72-hr culture in Dulaney's medium (pH 5.8). Agitation was at 250 rev/min for 120 hr at 30 C.
TABLE 2. Effect of ammonia nitrogen and naturally occurring nitrogen sources on L-lysine formation, growth, and sugar consumption of Ustilago maydis UV-ET-15a

| Sources                      | Optimal conc. (g/100 ml) | L-Lysine-HCl (g/liter) | Growth sugar consumption |
|------------------------------|--------------------------|------------------------|--------------------------|
|                              |                          |                        | G/100 ml | Per cent |
| Ammonium sulfate             | 0.30                     | 2.71                   | 0.91       | 66.8 |
| Ammonium acetate             | 0.20                     | 2.26                   | 0.94       | 68.4 |
| Diammonium phosphate         | 0.10                     | 2.53                   | 0.95       | 59.7 |
| Corn steep liquor            | 2.00                     | 1.87                   | 0.98       | 66.4 |
| Yeast extract                | 0.10                     | 0.77                   | 0.81       | 57.5 |

a A 50-ml amount of Dulaney's medium was placed in a 250-ml Erlenmeyer flask. A 1-ml amount of 1% corn oil was added. See footnote a, Table 1, for conditions.

TABLE 3. Effect of commercial carbon sources on L-lysine production by Ustilago maydis UV-ET-15a

| Carbon source                  | Medium | L-Lysine (mg/ml) | Growth (mg/ml) | Sugar consumption | Final pH % |
|--------------------------------|--------|------------------|---------------|-------------------|-----------|
| Sucrose                        | S-1    | 1.1-1.7          | 0.9           | 63.6              | 5.1       |
| Sucrose, hydrolyzed            | S-2    | 1.4-1.9          | 1.3           | 65.7              | 5.0       |
| Molasses                       | M-1    | 0.9-1.1          | 1.6           | 51.8              | 5.1       |
| Molasses treated               | M-2    | 0.9-1.3          | 1.7           | 57.1              | 5.8       |
| Molasses treated and hydrolyzed| M-3    | 1.0-1.3          | 2.0           | 63.7              | 5.7       |
| Clarified juice                | J-1    | 1.4-2.1          | 1.8           | 67.3              | 4.4       |
| Clarified juice, treated       | J-2    | 2.0-1.0          | 1.8           | 66.4              | 4.6       |
| Clarified and inverted juice   | J-3    | 1.5-2.4          | 2.1           | 67.1              | 4.5       |

a Fermentation medium was 26-b. See footnote a, Table 1, for conditions. Minimum and maximum values for L-lysine are from five experiments. Mean value is used for other determinations.

TABLE 4. L-Lysine production by Ustilago maydis UV-ET-15 in 7.5-liter fermentors

| Substrate | L-Lysine (g/liter) | Final pH |
|-----------|--------------------|----------|
| S-1       | 1.22-1.73          | 4.4-5.1  |
| M-1       | 0.90-1.37          | 4.0-5.1  |
| M         | 1.10-1.48          | 4.3-4.9  |
| J-1       | 1.73-2.10          | 4.0-4.8  |
| J-3       | 1.96-3.81          | 4.5-5.0  |

a A 5-liter volume, intitial pH 6.2, was agitated at 500 rev/min for 120 hr at 30 C. Aeration: 0.3 volume per volume per min. All media contained 1% corn steep liquor added. Value ranges taken from three tests.

TABLE 5. Kd values in 7.5-liter fermentors related to L-lysine production in medium J-3 by Ustilago maydis UV-ET-15a

| Kd | Agitation (rev/min) | L-Lysine (g/liter) | Mean viscosity (centipoises) | Mean density (g/ml) |
|----|---------------------|--------------------|------------------------------|---------------------|
| 7.25 | 300                 | 1.02-1.15          | 12.1                         |
| 12.00 | 700                 | 2.20-2.71          | 11.9                         | 1.05                |
| 1.22-1.90 | 700          | 11.6               | 1.04                         |

a Maximum and minimum values taken from three fermentations. Aeration: 0.5 volume per volume per min. Volume: 5 liters.

TABLE 4. L-Lysine production by Ustilago maydis UV-ET-15 in 7.5-liter fermentors

a Values to be multiplied by 10^4.

properly adjusted. The yields given in Table 4 show the slight variations observed with the best operating conditions. An increase in yield of 2.81 g of lysine per liter in medium J-3 (clarified and inverted juice) was reached at the end of 72 to 120 hr at 30 C, 500-rev/min agitation, and 0.3-volume per per min aeration.

To secure consistent results, it was estimated that a Kd × 10⁴ value of 12 gram-molecules of O₂ per liter per hr was apparently adequate for good lysine production in medium J-3 by the mutant under study (Table 5). The mean viscosity and density values of these fermentation broths were lower than those of the agave juice media (8). Further experiments suggest that a K₂ₕ value of 0.41 mole of O₂ per liter per min would permit more consistent results.

Some metabolic data of L-lysine formation in a 20-liter fermentor operated at a K₂ₕ value of 0.41 mmole of O₂ per liter per min are shown in
Table 6 and Fig. 1. The highest yield, 2.58 g of lysine per liter, was reached at the end of 72 hr coincident with rapid mycelial growth and low sugar consumption. The pH decrease paralleled carbohydrate utilization. Similar results were obtained in 7.5-liter fermentors. The lysine yields, by using mutant UV-ET-15 in the J-3 medium, are higher than those reported in the scientific literature and in some patents (Table 7) and are similar to those reached in agave juice media (8).

Finally, one of the fermentation broths obtained from a 100-liter fermentor with medium J-3 was evaporated and spray-dried; the powder obtained showed the chemical composition given in Table 8. As can be seen, the protein content is acceptable and the digestibility is good. Some of the main amino acids present in the concentrate are shown in Table 9.

**DISCUSSION**

Mutant UV-ET-15 of Ustilago maydis is a fair lysine producer. Yields of approximately 2.5 g per

**Table 6. Metabolic data of l-lysine formation in 20-liter fermentors with 10 liters of medium J-3 and Ustilago maydis UV-ET-15**

| Time (days) | Carbohydrate (% H) | Mycelium (mg/ml) | pH | L-Lysine (g/liter) |
|------------|---------------------|------------------|----|-------------------|
| 0          | 5.3                 |                  | 5.8| 1.25              |
| 2          | 5.0                 | 0.8              | 5.8| 1.25              |
| 3          | 4.1                 | 3.4              | 5.3| 2.58              |
| 4          | 3.7                 | 7.7              | 5.0| 2.46              |
| 5          | 3.3                 | 7.5              | 4.8| 2.51              |
| 6          | 2.8                 | 7.2              | 4.8| 2.57              |
| 7          | 2.2                 | 4.1              | 4.2| 2.61              |

*KLa* in the fermentors: 0.41 mmoles of O2 per liter per min. Temperature: 30 C; inoculum: 5%.

**Table 7. Published reports on yields of l-lysine by strains of Ustilago maydis**

| Strains | Substrate | L-Lysine (g/liter) | Time of fermentation (days) | Reference |
|---------|-----------|--------------------|----------------------------|-----------|
| NRRL 1229 | Cerelose-urea-mineral salts | 200-300 | 7 |
| PRL 1092  | Glucose-mineral salts | 300-600 | 5 | 3 |
| PRL 1704  | Glucose-mineral salts | 1,200-1,930 | 4-8 | 4 |
| PRL 1092  | Glucose-mineral salts | 145 | 5 | 4 |
| DC Cda  | Glucose-mineral salts | 300-500 | 10-12 | 5 |
| DC Cda  | Maltose-mineral salts | 380 | 3-5 | 4 |
| DC Cda  | Unhopped lager wort-distilled water | 310 | 5 | 6 |
| PRL 1092  | Glucose-mineral salts | 400 | 5 | 9 |
| PRL 1092  | Cane molasses (treated) | 12 | 3 | 9 |
| UV-ET-15  | Sugar cane clarified juice-corn steep liquor-corn oil | 1,700-2,800 | 3-4 | This paper |

* R. H. Kashins and J. F. T. Spencer, U.S. Patent No. 2,902,409; 1959.
Table 8. Analytical data of concentrate obtained from 50 liters of J-3 culture broth in 100-liter fermentors

| Determinations (hydrolyzed product) | Weight (%) |
|-------------------------------------|------------|
| Humidity                           | 10.80      |
| Protein                            | 14.70      |
| Fat                                | 2.23       |
| Fiber                              | 1.52       |
| Nifex                              | 80.75      |
| Ash                                | 8.19       |
| Digestibility                      | 86.10      |

Table 9. Amino acid composition of concentrate

| Amino acid     | Dry weight (g/liter) |
|----------------|----------------------|
| Hydroxyproline | 5.10                 |
| Lysine         | 2.90                 |
| Glutamic acid  | 1.76                 |
| Arginine       | 1.57                 |
| Serine         | 0.53                 |
| Aspartic acid  | 0.47                 |
| Threonine      | 0.33                 |
| Methionine     | 0.39                 |

liter can be obtained in clarified juice with the proper nitrogen source and agitation-aeration conditions controlled around a $K_{La}$ value of 0.41 mmole of $O_2$ per liter per min in the fermentors. The ammonium salts studied (phosphate, sulfate, and acetate) are equally good nitrogen sources for lysine production in this medium, depending on the concentration used. Contrary to observations of Kurtz and Ericson (5), ammonium acetate slightly increased the lysine yields as pointed out by other investigators (7, 8). The efficient utilization of the acetate would suggest a lysine biosynthetic pathway via ketoacidic acid (10). However, the addition of this acid or ketoacidic acid to the culture medium in concentrations as high as 10 mg/ml did not improve lysine production. We have no valid explanation for this fact.

The addition of corn steep liquor has a slight effect upon metabolite production but, according to previous observations (6, 8) and to our data (Table 2), this seems to be an indirect result of growth. Clarified juice could be utilized as a cheap carbon source for lysine production, because its local price is below that of cane sugar. The unsuitability of blackstrap molasses has been reported by Tauro et al. (9). However, our mutant grows well in this substrate and produces more lysine than the strain studied by these workers. Hydrolysis of cane molasses does not improve yields but can reduce the fermentation time from 120 to 72 hr since glucose is better assimilated than sucrose (Table 2).

By using the fermentation conditions recommended by several workers (4–7), unreplicable results, as they themselves have observed, were attained when commercial carbon sources were incorporated in the media. However, when the oxygen transfer was properly controlled, the variations in yield were not as wide as they appear from the results shown in Tables 4 to 6, which were obtained in fermentors of different sizes. $K_d$ values around 0.12 gram-molecule of $O_2$ per liter per hr or $K_{La}$ of 0.41 mmole of $O_2$ per liter per min allowed high and relatively constant results. Details of this study will be published elsewhere.

The maximum lysine value is reached between 48 and 72 hr during the initial growth period when the $pH$ is around 5.3 and the sugar consumption is low (Table 6; Fig. 1). At this time, sugar is apparently used for rapid mycelial growth and lysine reaches its highest value and remains constant, as does growth, until a period of 144 hr is reached. Afterwards, yields of lysine increase slightly as a probable result of mycelium lysis. Maximal lysine synthesis occurs when 11 to 20% sugar is consumed. Therefore, $pH$ and carbohydrate utilization, as well as growth and lysine synthesis, seem to be related as it was observed in the case of agave juice media (8).

Since preliminary experiments showed that the addition of specific minerals to the media (MgSO$_4$, MnCl$_2$, NaCl, FeSO$_4$, ZnSO$_4$, and CaCl$_2$), independently or together, did not affect lysine production even at a concentration of 0.2 g per liter, they were not added to the fermentation media. Probably the small amounts present in corn steep liquor are sufficient.

The chemical composition and the amino acid pattern of the lysine concentrate (Table 8, 9) suggest its utilization as a feed supplement. The vitamin B content of this concentrate has been previously reported (6, 8).

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