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Evaluation of the Potential of *Corynebacterium glutamicum* ATCC 21492 for L-Lysine Production Using Glucose Derived from Textile Waste

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Abstract: The textile industry generates millions of tons of waste annually, posing significant environmental challenges. Addressing this issue, our study explores a sustainable biotechnological approach to convert cotton textile waste into valuable bioproducts. We evaluated the potential of *Corynebacterium glutamicum* ATCC 21492 for the production of L-lysine and other amino acids using glucose derived from cotton textile waste. Two experimental strategies were implemented: Sequential Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF). In SHF, optimization of initial glucose concentration, temperature, and inoculum size led to the highest L-lysine concentration of 2.38 g/L under conditions of 45 g/L glucose, 35 °C, and 2% inoculum. The production of L-lysine, along with varying proportions of other amino acids such as alanine, threonine, methionine, and leucine, was significantly influenced by these parameters. In SSF, the highest L-lysine yield of 3.10 mg/g untreated cotton was achieved at 14% cotton loading, 7% enzyme dose, 35 °C, and 10% inoculum concentration, corresponding to an L-lysine concentration of 0.5 g/L. This reduced concentration, compared to SHF, is primarily attributed to limitations in cotton hydrolysis under the studied conditions. Nevertheless, *C. glutamicum* utilized alternative carbon sources present in the culture medium, leading to a diversified amino acid profile in the final product. These findings support the feasibility of textile waste bioconversion using *C. glutamicum* and highlight its potential as a sustainable platform for amino acid production, aligning with circular economy principles and contributing to the reduction of the textile industry's environmental impact.

Keywords: enzymatic hydrolysis; fermentation; SHF; SSF; circular economy; sustainable production; biotechnological valorization

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1. Introduction

The textile industry, driven by the "fast fashion" model, has grown significantly in recent decades, increasing textile waste production and its environmental impact. Ninety-two million tons of textile waste are generated annually, of which only a minimal fraction is recycled [1–3]. This issue is exacerbated by the use of synthetic fibers, chemical dyes, and processes that consume large amounts of water and energy. In 2016, the textile industry emitted approximately 4 Gt of CO_2, representing 8% of global greenhouse gas emissions [4]. Additionally, washing synthetic garments generates 35% of oceanic microplastics, and an estimated 215 trillion liters of water are consumed annually in this industry [5]

The circular economy emerges as a solution to mitigate this impact through textile recycling strategies. Mechanical recycling, while cost-effective, reduces fiber quality, limiting reuse potential [6]. On the other hand, chemical recycling enables the recovery of high-quality fibers but requires advanced technologies and high operational costs [6,7]. A promising alternative could be the use of biorefinery processes, using cellulose from textile waste as a carbon source for the biotechnological production of biopolymers, bioethanol, and essential amino acids. This process involves enzymes that hydrolyze cellulose into glucose, which is then fermented by microorganisms such as *Saccharomyces cerevisiae* and *Corynebacterium glutamicum* [8,9].

L-lysine, an essential amino acid for the food and pharmaceutical industries, stands out in this context. It has been demonstrated that lignocellulosic materials can be a viable source for L-lysine production via fermentation, particularly using genetically modified microorganisms like *C. glutamicum* [10–12]. As an alternative, cellulose from textile waste could reduce the need for pretreatment processes, i.e., delignification. This approach reduces dependence on agricultural resources and promotes waste reuse.

In this study, two main strategies were evaluated for converting cellulosic textile waste into value-added products: Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF). The SHF process allows the independent optimization of enzymatic hydrolysis (EH) and fermentation stages to maximize efficiency, while SSF combines the enzymatic hydrolysis of cellulose and the fermentation process into a single step. This integration not only simplifies the overall process by reducing the need for separate optimization and handling of intermediate products but also has the potential to lower operational costs by minimizing equipment requirements, reducing processing time, and improving overall efficiency.

The results demonstrate that converting textile waste into L-lysine through these processes is a sustainable alternative to traditional sources of this amino acid. Moreover, it provides a solution for managing textile waste, contributing to the circular economy, and mitigating the environmental impact of this industry [13].

2. Materials and Methods

2.1. Cotton Sample: Source and Composition Analysis

The raw material used in this study was kindly provided by Recover Fiber®, a byproduct of their production of recycled yarn from pre-consumer textile waste. Specifically, the sample is 100% undyed cotton, the fibers of which are too small to be re-spun.

The structural characterization of the cotton sample was carried out after milling using an ultra-centrifugal mill (Retsch ZM200, Haan, Germany) with a 0.5 mm sieve. Cellulose, hemicellulose, and lignin contents were analyzed using the Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), and crude fiber (Weende) methods, employing digestion with H_2SO_4 and NaOH. These techniques have been widely used in previous studies [14–16]. Acid Detergent Lignin (ADL) was quantified after treatment with 72% H_2SO_4 followed by calcination.

2.2. Pretreatment of Cotton Biomass

Physical pretreatments were evaluated to enhance cellulose degradability, including milling with an ultra-centrifugal mill (Retsch ZM200, 0.5 mm sieve) for homogenization and particle size reduction, and ultrasonic treatment (UIP1000, Hielscher Ultrasonics, Rehovot, Israel, 20 kHz, 1000 W, 5 min at maximum power) to reduce cellulose crystallinity.

2.3. Enzymatic Hydrolysis Protocol

To perform the enzymatic hydrolysis (EH) of cotton, the commercial blend Cellic® CTec2 at a dose of 0.24 mL/g of cotton (Novozymes, Frederiksberg, Denmark) was initially used, as it is widely cited in the literature [17]. This blend contains cellulases, ß-glucosidases, and hemicellulases for cellulose degradation into fermentable sugars. In the course of the present study, Cellic® CTec2 was discontinued and, thus, other alternatives (Novozymes, Denmark) were tested: Fibercare® D, Saczyme®Yield, Flavourzyme®, Cellic® CTec3, Fiberlife® 550, and Fiberlife® 500. These alternatives exhibited a similar enzymatic composition compared to Cellic® CTec2.

The optimal pH for Cellic® Ctec2, Fibercare® D, Saczyme®Yield, Flavourzyme®, and Cellic® CTec3 is 4.8, for which a citrate buffer (0.1 M sodium citrate ($C_6H_8O_7 \bullet H_2O$; Labkem, Barcelona, Spain) at 60% and 0.1 M citric acid ($C_6H_5O_7Na_3 \bullet 2H_2O$; Labkem) at 40% was used. Fiberlife® 500 and 550 operate better at pH 6.6, for which deionized water was used as the liquid medium.

The EH of cotton to obtain fermentable carbohydrates was performed at 50 °C and 200 rpm, following the specifications provided by the manufacturer (Novozymes, Denmark). To determine the optimal process conditions, hydrolysis was conducted using different proportions of both pretreated and untreated cotton (Table 1) with samples collected at 72, 96, 120, and 144 h. All experiments were conducted in at least triplicate.

Table 1. Enzymatic hydrolysis (EH) tests on cotton at different concentrations and pretreatments performed to obtain fermentable carbohydrates.

Test Name	Pretreatment	Cotton Dose (%)	Duration of Test (h)
EH1	None	2.5	144
EH2	Milling	2.5	144
EH3	Milling + Ultrasounds	2.5	144
EH4	Blank: Milled cotton + buffer	2.5	144
EH5	Control: Buffer	0.0	144
EH6	None	10.0	120
EH7	Milling	10.0	96
EH8	None	20.0	96
EH9	Milling	20.0	96
EH10	None	12.5	96
EH11	None	15.0	96
EH12	None	17.5	96

To ensure homogeneous wetting of the cotton and minimize variability, the buffer was gradually added under constant manual stirring. Reaction flasks were then sealed and incubated at 50 °C and 200 rpm for 72 h in a Labolan Mod 200 orbital shaker (Lan Technics, Madrid, Spain).

Additionally, to establish the optimal conditions for L-lysine production from cotton waste via Simultaneous Saccharification and Fermentation (SSF), hydrolysis experiments were conducted to evaluate enzyme performance in the fermentation medium required by the bacterial strains. These tests were performed in the presence or absence of 0.1 M citrate buffer or $CaCO_3$ (25 g/L) at pH values of 4.8 and 6.6. The conditions of these experiments are detailed in Table 2, with a cotton concentration of 20%.

Table 2. EH tests on 20% cotton dose in culture media and 2.4 mL/g of cotton of Cellic® Ctec2.

Test Name	Liquid Media	pH
EH13	Fermentation media	Adjusted to 4.8
EH14	Fermentation media on citrate buffer [1]	4.8
EH15	Fermentation media + $CaCO_3$ [2]	Adjusted to 6.6
EH16	Fermentation media on citrate buffer + $CaCO_3$ [2]	6.6

[1] Citrate buffer 0.1 M. [2] $CaCO_3$ concentration: 25 g/L.

During the execution of this study, the Cellic® CTec2 cellulase preparation was no longer available, necessitating the optimization of hydrolysis conditions using new commercial enzyme preparations. The last EH tests (EH17-EH25) were performed with the alternative commercial enzyme preparations (Table 3). All experiments were conducted for 72 h under the same temperature (50 °C) and agitation (200 rpm) as those performed with Cellic® CTec2. Cotton dose was 20% *w*/*w* in all test, with the exception of test EH19, which was 14% *w*/*w* cotton. pH and enzyme concentrations were applied according to the manufacturer's recommendations, except for experiments EH24 and EH25, where higher and lower concentrations than the recommended dosage were tested for the Fiberlife® 550 enzyme preparation.

Table 3. EH tests with the alternative enzymatic blends and different cotton doses and pH.

Test Name	Enzymatic Blend	Dose (g Blend/g Cotton)	pH
EH17	Fibercare® D	0.0003	4.8
EH18	Saczyme®Yield (20% *w*/*w* cotton)	0.5	4.8
EH19	Saczyme®Yield (14% *w*/*w* cotton)	0.5	4.8
EH20	Flavourzyme®	0.166	4.8
EH21	Cellic® CTec3	0.25	4.8
EH22	Fiberlife® 550	0.07	6
EH23	Fiberlife® 500	0.07	6
EH24	Fiberlife® 550	0.2	6
EH25	Fiberlife® 550	0.01	6

Next, the hydrolysates were filtered using a vacuum pump (N 840 Laboport, Freiburg, Germany), 1 L Erlenmeyer flasks, and 110 mm Prat Dumas qualitative analysis filters. Once hydrolysates from different cotton pretreatments were obtained, an aliquot was taken to determine the total carbohydrate concentration via ion chromatography.

2.4. Microbial Strain, Media Composition, and Cultivation Conditions

For the L-lysine production from textile residues, a strain of *C. glutamicum* ATCC 21492, genetically modified using the Ajinomoto Co., Inc. (Tokyo, Japan) protocol [18], was used. This strain was provided by the MATERIA NOVA Innovation Center. As observed by several authors [19–21], a partial redirection of *C. glutamicum* metabolism toward the synthesis of other amino acids, such as alanine and methionine, is expected.

The microorganism was reconstituted from frozen stocks (−80 °C) by inoculating in a medium consisting of 15 g/L of commercial tryptic soy broth (TSB) supplemented with 25 g/L glucose [13,22,23]. The commercial TSB was composed of casein, 17 g; soya peptone, 3 g; sodium chloride, 5 g; dipotassium hydrogen phosphate, 2.5 g; glucose, 2.5 g. Five milliliters of prepared medium (TSB supplemented with 25 g/L of glucose) was inoculated in 50 mL Erlenmeyer flasks and incubated at 30 °C, 120 rpm, for 24 h in an orbital incubator (LABOLAN Mod 200). Serial dilutions were prepared (10^{-1} to 10^{-7}) and plated on TSA (15 g/L) supplemented with 25 g/L glucose. Plates were incubated at 30 °C for 24 h in an ARGOLAB TCN-115 incubator (Argo Lab, Carpi, Italy).

Subsequently, precultures were obtained by inoculating 5 mL of liquid TSB medium in 25 mL Erlenmeyer flasks with a single isolated colony from the TSA solid medium, as described above. These cultures were incubated for 24 h at 30 °C and 120 rpm in an orbital shaker.

All culture media used were sterilized in a SELECTA Presoclave-III 80 L (J.P.SELECTA, Barcelona, Spain) autoclave at 120 °C for 45 min. After the incubation period, the preculture was ready for use as an inoculum in subsequent fermentations.

Fermentation was conducted in the basal medium containing: magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 2.25 g/L; iron sulfate ($FeSO_4 \cdot 7H_2O$), 11.25 g/L; magnesium chloride ($MgCl_2 \cdot 6H_2O$), 11.25 g/L; hydrolyzed casein, 6.25 g/L; ammonium sulfate ($(NH_4)_2SO_4$), 31.25 g/L; anhydrous potassium hydrogen phosphate (K_2HPO_4), 1.25 g/L; calcium carbonate ($CaCO_3$), 25 g/L; thiamine, 0.2 mg/L; biotin, 0.2 mg/L; threonine, 240 mg/L; methionine, 330 mg/L dissolved in distillated water.

2.5. Experimental Design for Growth Modeling of C. glutamicum

Growth Phase on Plate. To characterize the growth of the *C. glutamicum* ATCC 21492 strain, the concentration of colony-forming units per milliliter (CFU/mL) was determined, allowing for the modeling of population dynamics over time. The protocol described for preculture preparation was followed, and the growth assay was performed in triplicate using 50 mL flasks over a period of 36 h at 30 °C, 120 rpm, and a working volume of 5 mL in an AGROLAB TCN 115 incubator.

The CFUs were counted following the standard range of 25 to 300 colonies per plate, as this range ensures statistical accuracy and prevents colony overlap, as established by NOM-092-SSA1-1994 (DOF; UV, 2020).

Bacterial Growth Modeling. Several growth models were tested to assess their ability to predict population size (CFU/mL) over time. To select the most suitable model, parameters were optimized by minimizing the root mean square error (RMSE) using the curve-fit algorithm and grid search.

The selected model was the modified Gompertz model, which is expressed as follows:

$$ln\frac{N(t)}{N_0} = A \cdot \exp(-\exp\left(\frac{\mu_{max} \cdot e}{A} \cdot (\lambda - t) + 1\right))$$

where $N(t)$ is the CFU value (CFU/mL) for a given time and N_0 the CFU value (CFU/mL) at the initial time; A is the asymptote (ln [$N\infty/N_0$]) in the stationary phase; μ_{max} is the maximum specific growth rate (h^{-1}), which comes expressed as $(\ln(CFU_t) - \ln(CFU_{t-1}))/(t_t - t_{t-1})$; e is the Euler's number; λ is the lag time (h) and t is the time (h).

2.6. Fermentation Test Using Commercial Glucose as Carbon Source

Fermentations were conducted at 70 g/L initial glucose, 30 °C, 2% (*v*/*v*) inoculum, pH 7.5, and 120 rpm in 0.5 L flasks with a working volume of 25 mL. Samples were taken daily for amino acid quantification. Additional fermentations were performed at

varying glucose concentrations (10–100 g/L) to assess substrate inhibition under identical conditions. Additionally, specific tests were conducted by inoculating with a 2% and 10% v/v preculture and increasing the temperature to 35 °C and 37 °C, selecting the initial glucose concentration in which the SHF assays would be performed, i.e., 35 g/L and 50 g/L. At 35 g/L, the central point of the SHF study, all the combinations were tested.

2.7. Sequential Hydrolysis and Fermentation (SHF): Experimental Setup

The SHF fermentation stage followed a protocol similar to that used for commercial glucose fermentations, but prepared fermentation basal medium by adding the necessary nutrients directly into the different hydrolysates, rather than using water as the solvent. Preculture preparation for inoculation was performed as previously described.

The experimental design considered three variables: initial glucose concentration (24, 33, and 45 g/L), temperature (30 and 35 °C), and inoculum size (2 and 10% v/v). The glucose concentrations tested were based on the optimization of the EH and previous fermentation assays performed in the present research with commercial glucose. Specifically, the 45 g/L glucose concentration was obtained under the conditions of the EH11 assay, the 33 g/L concentration from the EH6 or EH18 assays, and the 24 g/L concentration from the EH19 assay.

Prior to inoculation, all fermentation media were supplemented with vitamins and adjusted to pH 7.5. Then, 25 mL of the prepared medium was transferred to 250 mL Erlenmeyer flasks and inoculated accordingly. Fermentations were carried out at the selected temperatures and 120 rpm for 72 h. After incubation, samples were collected in duplicate from each flask for further analysis.

2.8. Simultaneous Saccharification and Fermentation (SSF): Experimental Setup

SSF was carried out by treating the cotton substrate simultaneously with the enzymatic cocktail and the *C. glutamicum* inoculum. For this purpose, a liquid mixture was prepared containing the selected enzyme preparation (Fiberlife® 550) and the bacterial inoculum dissolved in the basal medium. This mixture was added to the cotton according to the solid-to-liquid (S:L) ratio established for each condition, following the same procedure used in the enzymatic hydrolysis assays.

The flasks were incubated in an orbital shaker at 200 rpm for 72 h, maintaining the specific temperature for each assay.

For the experimental design, four parameters at two levels were evaluated: cotton load (14 and 20%), enzyme dose (5 and 7%), temperature (30 and 35 °C), and inoculum concentration (2 and 10%). Therefore, a full factorial design (2^4) was implemented, resulting in 16 experimental conditions encompassing all possible parameter combinations. In addition, blank assays were performed at a 10% cotton dose, varying the inoculum size and temperature. All assays were performed in triplicate to ensure reproducibility.

2.9. Analytical Techniques for Sugar and Amino Acid Quantification

Glucose quantification was performed by ion chromatography using a Metrohm system equipped with a pulsed amperometric detector (PAD) for the detection of sugars and polyols. The system included a chromatographic module with an injection valve, temperature control, a high-pressure isocratic pump, eluent organizer, carbohydrate analysis column with guard column, and an autosampler.

L-lysine produced by *C. glutamicum* strains, as well as other amino acids, were analyzed by high-performance liquid chromatography (HPLC). An Agilent 1290 Infinity II LC system (Santa Clara, CA, USA) was used, equipped with a quaternary pump (G7104A), standard autosampler (G7167B), column oven (G7116B), and a fluorescence detector (1260 FLD Infinity II). Compound separation was achieved using a reverse-phase ZORBAX

Eclipse Plus C18 column (Agilent, Santa Clara, CA, USA) (3.0 × 100 mm; 1.8 µm) with a ZORBAX RRHD Eclipse Plus C18 guard column (1.8 µm, 3.0 mm, 1200 bar, UHPLC).

2.10. Statistical Treatment of Data

The influence of inoculum size and temperature on the L-lysine yield was determined by means of an analysis of variance (ANOVA) with a p value lower than 0.05. Dixon's Q test was used to estimate and reject outlier values using a confidence level of 95%.

3. Results

3.1. Physicochemical Characterization of Cotton Residues

Table 4 presents the structural characterization of the cotton sample. The results indicate that the cotton is of high quality for biotechnological applications. Its substantial cellulose content (92.0% dry basis) confirms that it is predominantly composed of structural polysaccharides, making it suitable for enzymatic hydrolysis and fermentation processes. Furthermore, the low lignin content (4.02% dry basis) suggests that the material's structure does not present a significant barrier to enzymatic action, thereby facilitating the release of fermentable sugars.

Table 4. Cotton characterization.

Parameter	Value (d.b.) (%)
Moisture	4.51
Cellulose	92.0
Hemicellulose	5.71
Acid Detergent Lignine	4.02

3.2. Enzymatic Hydrolysis Performance

Figure 1 shows the glucose concentrations obtained in experiments EH1-EH5, focusing on the influence of pretreatments on the enzymatic hydrolysis of cotton with a solid-to-liquid ratio of 2.5%. The milled cotton (EH2) achieved slightly higher glucose concentrations compared to the untreated sample (EH1). On the other hand, the application of ultrasound significantly reduced the hydrolysis yield, leading to the exclusion of this treatment from future experiments. The glucose concentration achieved in experiments EH4 and EH5 (control and blank, respectively) was negligible, confirming that, in the absence of enzymes, cotton does not degrade into glucose, as expected.

Analyzing the reaction's progression over time, a duration of 72 h was chosen for enzymatic hydrolysis experiments. Although the maximum glucose value is nearly reached at 120 h, the difference between 72 and 120 h is minimal, especially in the milled sample. Additionally, no significant improvement in yield was observed at 96 h. Considering the cellulose content in cotton (Table 4), the hydrolysis yield for untreated cotton is 0.38 g glucose/g cellulose at 72 h, reaching a maximum of 0.499 at 120 h. The milled cotton approaches 0.49 g glucose/g cellulose at 72 h, while at 120 h, it is 0.504 g glucose/g cellulose.

In a subsequent series of experiments, additional cotton concentrations of 10% and 20% w/v S:L were evaluated using both the original and milled samples (experiments EH6–EH9), with results presented in Figure 2. Given that milling did not significantly enhance hydrolysis and considering the increased process costs associated with this pretreatment, it was deemed unnecessary. Consequently, experiments EH10–EH12 were conducted with untreated cotton at intermediate concentrations of 12.5%, 15%, and 17.5%. A nearly linear relationship was observed between cellulose concentration and generated

glucose, ranging from 32.92 g/L to 60.17 g/L for 10% and 20% cotton concentrations, respectively. Hydrolysis yields were 0.351 g glucose/g cellulose for 10% cotton and 0.327 g glucose/g cellulose for 20%, indicating a decrease in yield with increasing S:L ratio.

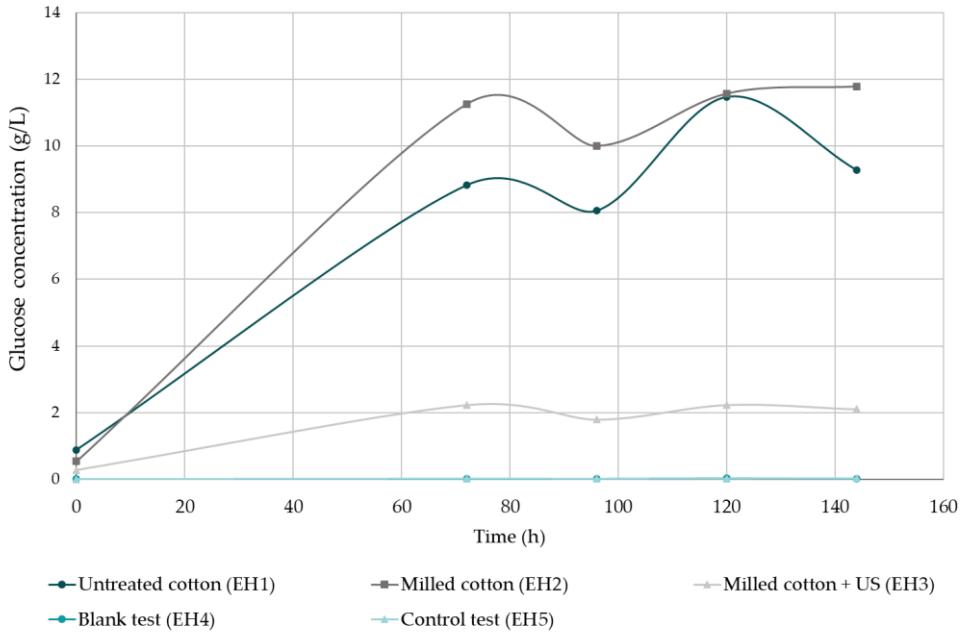


Figure 1. Glucose concentration vs. time obtained with the EH of pretreated and untreated cotton at a 2.5% dose.

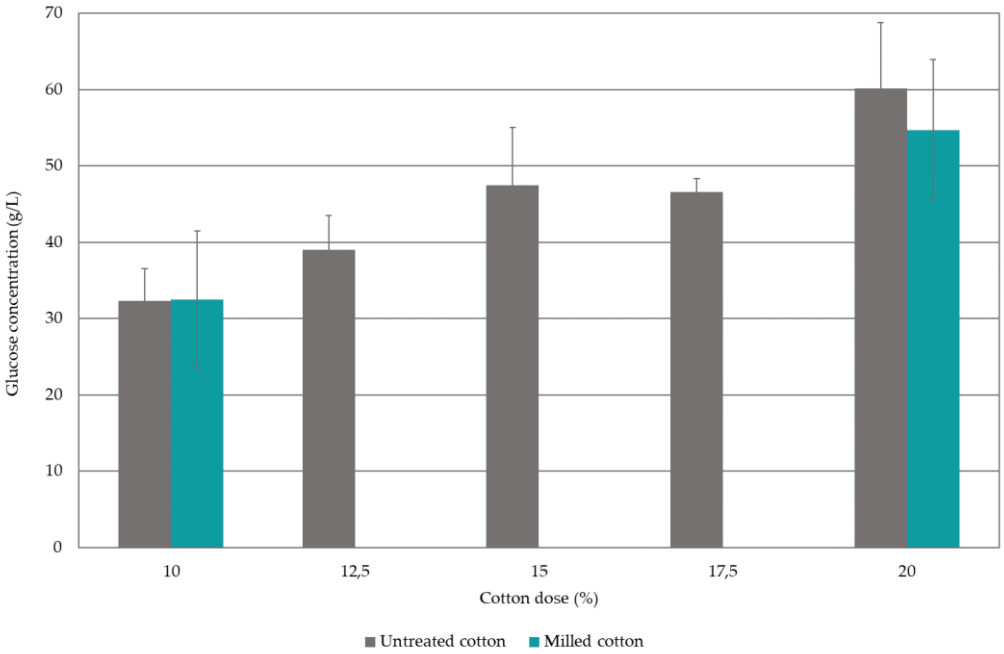


Figure 2. Glucose concentration achieved with different cotton doses after 72 h of EH.

Experiments modifying the reaction medium (Figure 3) demonstrated that the presence of nutrients required for bacterial cultivation negatively impacted process efficiency. Additionally, increasing the pH to 6.6 significantly reduced the hydrolysis yield.

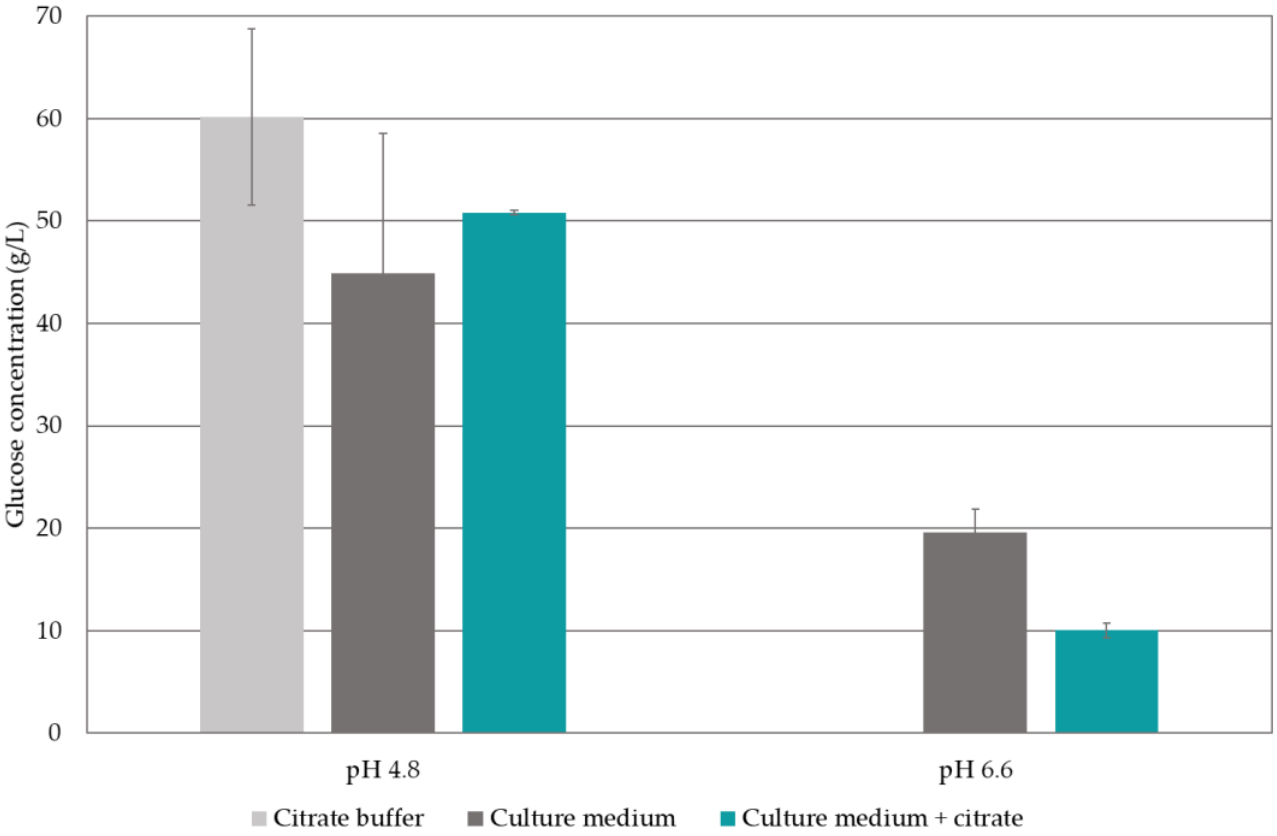


Figure 3. Glucose concentration achieved after 72 h of EH in different liquid media.

Table 5 presents the hydrolysis results of the alternative enzyme blends under conditions of 20% *w*/*w* cotton dose, 50 °C, and 200 rpm agitation for 72 h. Enzyme concentrations followed manufacturer recommendations, except in experiments testing higher and lower doses of Fiberlife® 550. The findings indicate that only the Saczyme®Yield and Fiberlife® 550 blends (the latter at the recommended concentration) achieved glucose concentrations comparable to Cellic® CTec2. Specifically, Saczyme®Yield produced 31.06 g/L of glucose, and Fiberlife® 550 achieved 36.92 g/L after 72 h. In contrast, deviating from the recommended Fiberlife® 550 concentration drastically reduced yield, underscoring the importance of adhering to the specified dosage.

Table 5. Glucose concentration (g/L) after 72 h of the EH tests with the alternative enzymatic blends and different cotton doses and pH.

Test Name	Enzymatic Blend	Glucose Concentration
EH17	Fibercare D	0.31
EH18	Saczyme®Yield (20% *w*/*w* cotton)	31.06
EH19	Saczyme Yield®(14% *w*/*w* cotton)	24.02
EH20	Flavourzyme®	3.82
EH21	Cellic® CTec3	4.01
EH22	Fiberlife® 550	36.92
EH23	Fiberlife® 500	0.98
EH24	Fiberlife® 550	0.54
EH25	Fiberlife® 550	1.50

3.3. Modeling of C. glutamicum Growth Dynamics

In fermentations, the bacterial growth can be defined by three phases: lag, exponential, and stationary phase. The maximum bacterial population is reached in the latter phase. The strain growth was determined to standardize the bacterial population in the inoculum, which was carried out in TSB supplemented with 25 g/L of glucose at 30 °C, 120 rpm, for 24 h. Figure 4 illustrates the growth dynamics of *C. glutamicum* ATCC 21492 measured in CFU/mL. Under the growth conditions, the *C. glutamicum* strain had a lag phase of approximately 6 h (λ = 6 h), reaching an average population of about 3.5×10^6 CFU/mL. After this fermentation time, there was a rapid increase in cell density up to 21 h (exponential phase). The bacterial population rose from 3.5×10^6 to 1.9×10^9 CFU/mL at 24 h. During the exponential phase, the maximum specific growth rate (μ_{max}) was reached: 0.8317 h^{-1}, which was obtained between 6 and 9 h of fermentation.

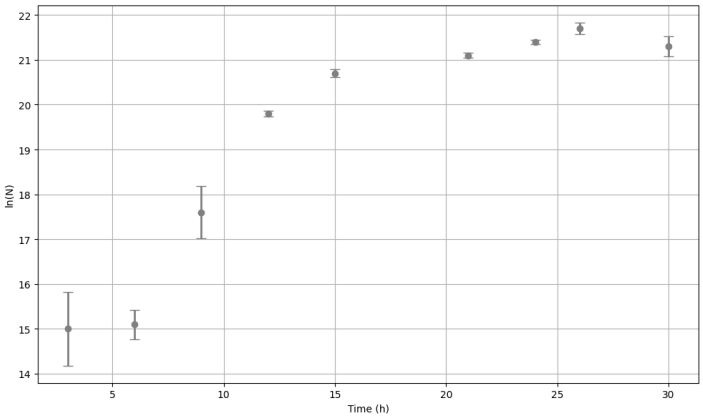


Figure 4. Growth trend of *C. glutamicum* ATCC 21492.

Nevertheless, the bacterial growth continued to rise, peaking at 26 h with a concentration of 2.73×10^9 CFU/mL (Figure 4).

Figure 5 illustrates the various mathematical models evaluated in this study. Based on the data, Gompertz and modified Gompertz models yielded the highest R^2 (0.9963), being the most suitable for describing the growth of *C. glutamicum*. Among these, the modified Gompertz curve was selected for estimating the growth of the *C. Glutamicum* ATCC 21492 strain as follows:

$$ln\frac{N(t)}{N_0} = 6.3232 \cdot \exp(-\exp\left(\frac{0.8317 \cdot e}{6.3232} \cdot (5.9921 - t) + 1\right))$$

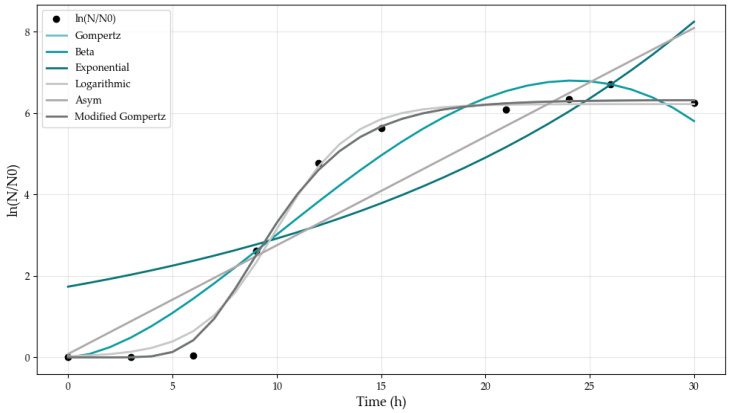


Figure 5. Growth of *C. glutamicum* according to the different mathematical models studied.

3.4. L-Lysine Fermentation Using Commercial Glucose

Figure 6 presents a bar graph illustrating the L-lysine production yield relative to the initial glucose concentration under various experimental conditions, while Figure 7 depicts the final glucose concentration. Notably, the lowest and highest glucose concentrations tested (10 and 100 g/L) resulted in suboptimal L-lysine production. Optimal production at 30 °C appears to occur with initial glucose concentrations between 25 and 70 g/L, as moderate glucose levels optimize metabolic efficiency and avoid inhibitory effects observed at extreme concentrations. Similar results have been reported in previous studies [13,22–25].

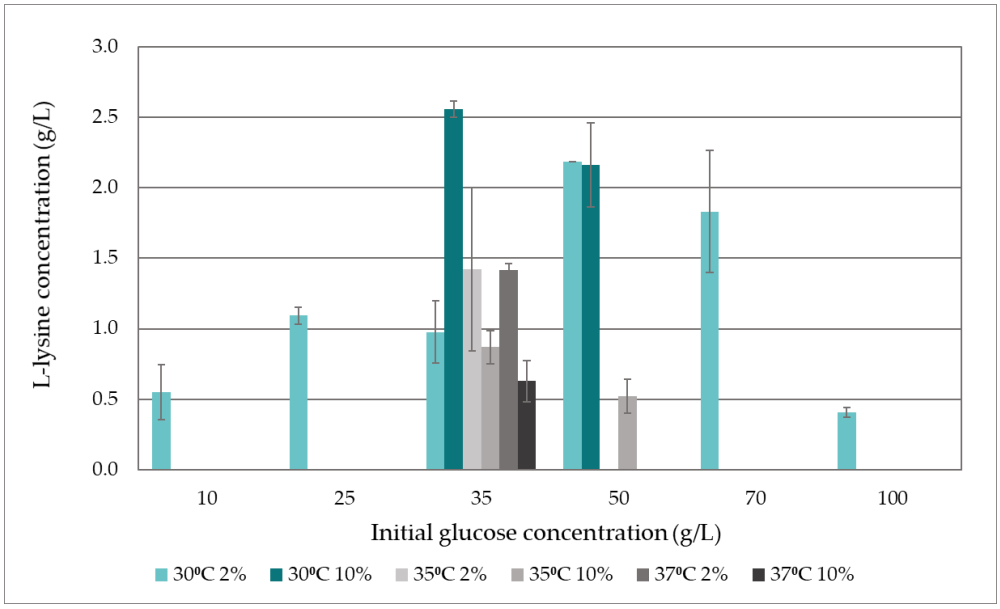


Figure 6. L-Lysine concentration achieved after 72 h fermentation with *C. Glutamicum* starting from different initial glucose concentrations in synthetic media.

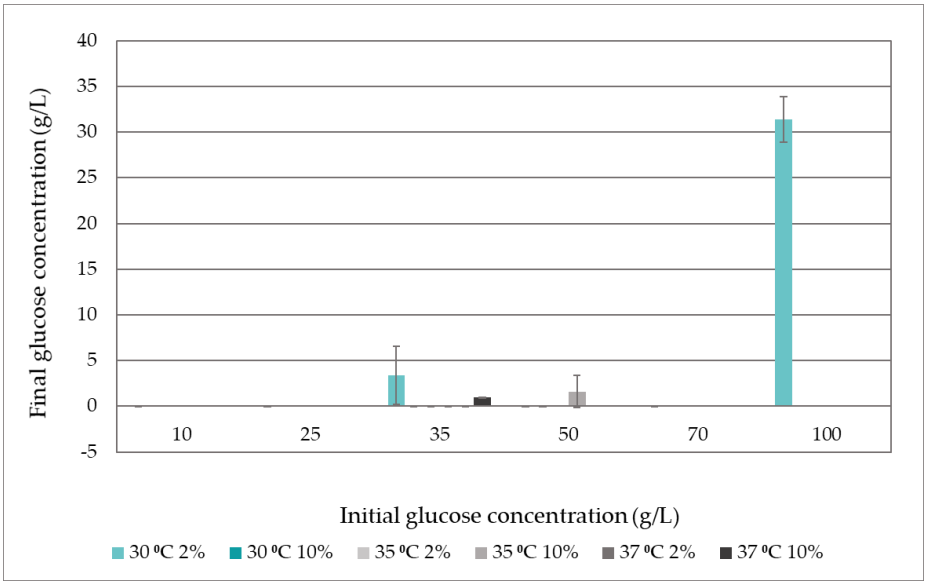


Figure 7. Residual glucose concentration after 72 h fermentation with *C. Glutamicum* starting from different initial glucose concentrations in synthetic media.

Increasing the fermentation temperature to 35 °C and 37 °C adversely affected L-lysine production, particularly at higher inoculum concentrations. Among the conditions tested,

only at 30 °C with moderate initial glucose levels did higher inoculum concentrations enhance L-lysine production.

Regarding residual glucose concentration, nearly complete utilization of glucose was observed within 72 h, except in the experiment with an initial glucose concentration of 100 g/L, suggesting substrate inhibition at this higher concentration.

The concentration of 35 g/L was selected to study the influence of the combination of temperature and inoculum dose on the L-lysine yield. A two-way ANOVA revealed that both temperature and inoculum dose had a significant effect on L-lysine concentration at 35 g/L glucose, with a highly significant interaction between factors ($p < 0.0001$).

Based on these results, the optimization of key variables, such as inoculum concentration, temperature, and initial glucose concentration, was carried out in fermentations using cotton hydrolysate as the carbon source. Furthermore, this study established the range of initial glucose concentrations to be considered in the subsequent experimental phase.

3.5. SHF Performance

Figures 8 and 9 show the influence of initial glucose concentration, temperature, and inoculum dose on L-lysine production by *C. glutamicum* ATCC 21492 and on the residual glucose concentration, respectively. It can be seen that L-lysine production increased with the initial glucose load, being minimal at 24 g/L. In contrast, 33 g/L resulted in a significant increase, and 45 g/L yielded the highest values, although the difference between 33 and 45 g/L was less pronounced than that between 24 and 33 g/L, suggesting a possible saturation of the system. On the other hand, a positive effect in L-lysine production was observed when working at 35 °C compared to 30 °C in most conditions, particularly at 45 g/L glucose.

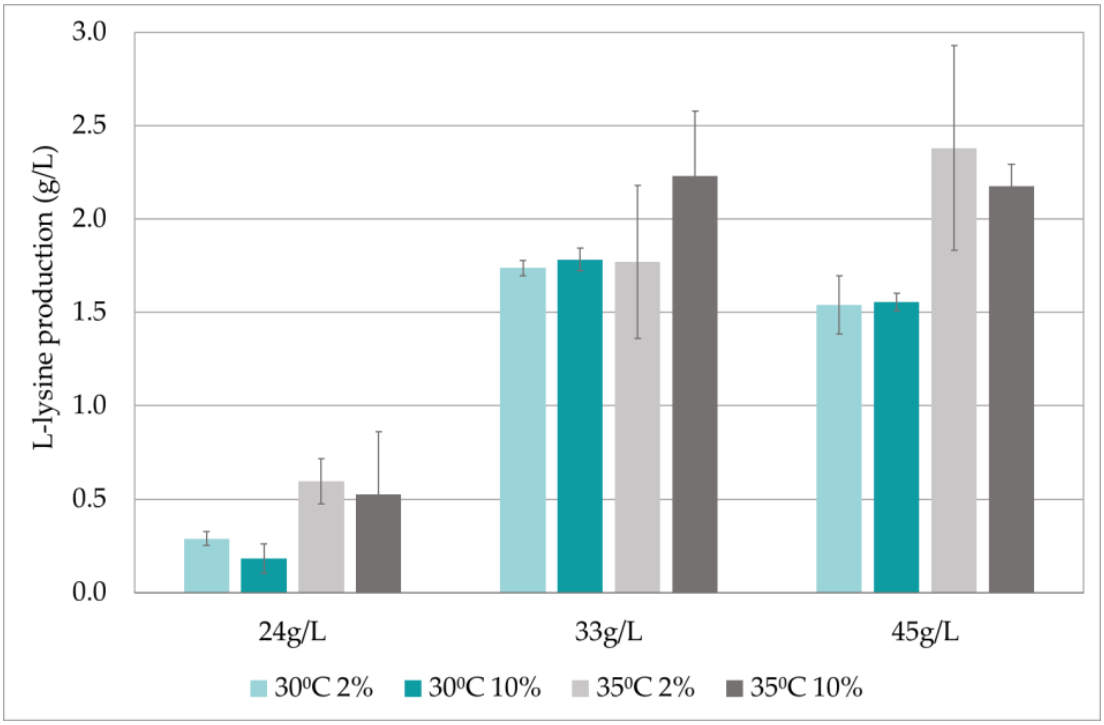


Figure 8. L-lysine concentration after 72 h fermentation of different glucose concentration hydrolizates by *C. Glutamicum* ATCC21492.

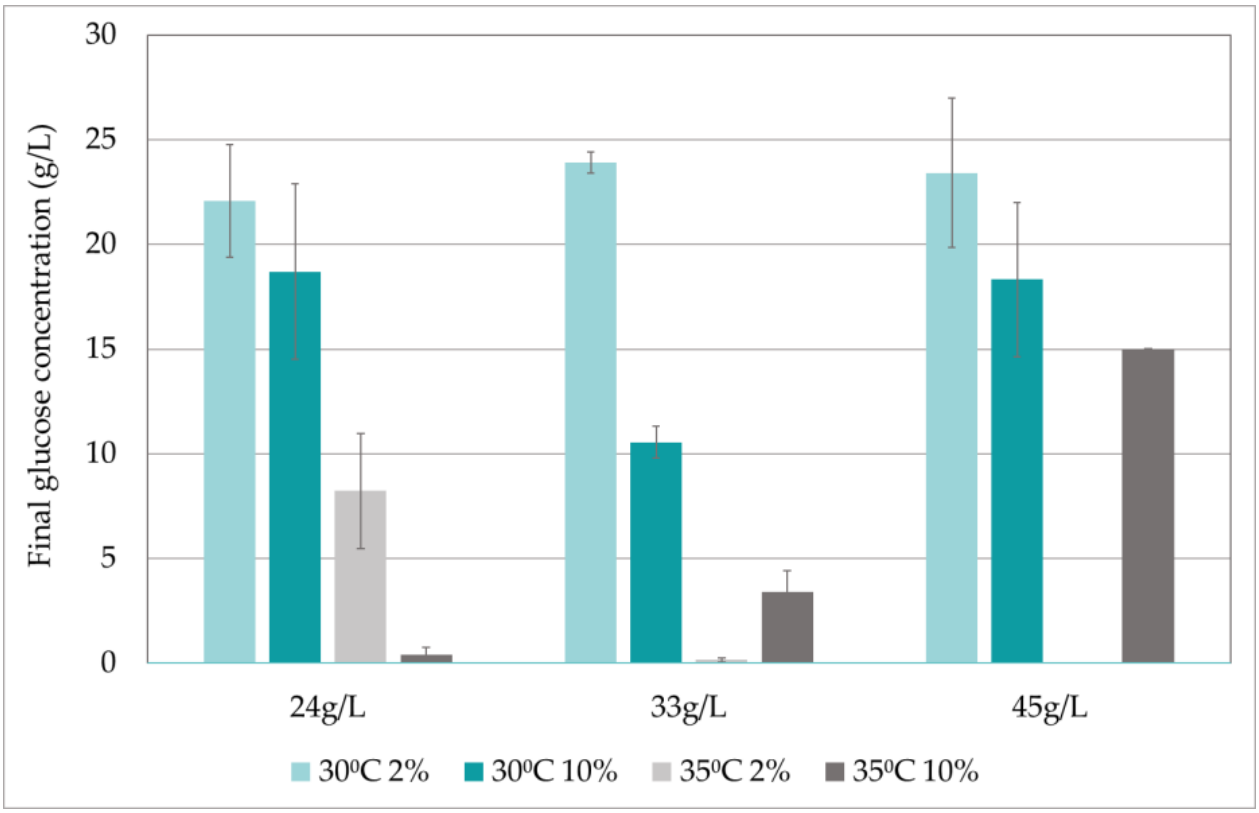


Figure 9. Residual glucose concentration after 72 h fermentation of different glucose concentration hydrolizates by *C. Glutamicum* ATCC21492.

An ANOVA was performed, which revealed that glucose, temperature, and inoculum dose significantly influence L-lysine production ($p < 0.05$). Among these factors, glucose stood out as the most decisive, exerting the greatest direct effect on production. Furthermore, the interactions between factors were also significant, indicating that the relationship among glucose, temperature, and inoculum dose plays an important role in the process.

The influence of inoculum size on L-lysine production exhibited notable variability, contingent upon both the initial glucose concentration and the fermentation temperature. Specifically, as seen before using commercial glucose as a carbon source, higher substrate concentrations and a lower temperature (30 °C), increasing the inoculum size, resulted in higher L-lysine titers, but this time, this effect was less notable. Conversely, working at 35 °C inverted this trend. An exception was observed in the assay conducted at 35 g/L glucose, 35 °C, and 2% inoculum, where L-lysine production did not follow the general trend. However, this particular assay exhibited a notably high standard deviation, as shown by the error bars, precluding definitive conclusions regarding the observed trend. Furthermore, in experiments utilizing commercial glucose under the same conditions (35 g/L glucose and 35 °C), an increased inoculum size also resulted in reduced L-lysine yields. This consistency across different carbon sources reinforces the observed pattern and suggests that the exception noted may be attributed to experimental variability rather than a distinct biological response.

It is noteworthy that the precultures used for fermentation had an initial concentration of $1.9 \times 10^9 \pm 6.68 \times 10^8$ CFU/mL and were collected at 24 h, a time point at which the bacterial population may be transitioning between exponential growth and the stationary phase. Minor variations in the timing of preculture preparation prior to inoculation could significantly affect the bacterial population density, due to the high growth rate characteristic of the exponential phase. This variability in initial cell density could contribute to

the high standard deviation observed in experiments evaluating the influence of different inoculum doses in the fermentation medium, both in fermentations using commercial glucose and in those utilizing cotton hydrolysates.

Similarly, regarding glucose consumption by *C. glutamicum* ATCC 21492, high temperature and inoculum size favored higher yields under low-glucose conditions, whereas the optimal combination at high substrate levels was 45 g/L glucose, 35 °C, and 2% inoculum, where residual glucose was zero.

Additionally, as expected, HPLC chromatograms revealed the presence of amino acids other than L-lysine, such as alanine, glutamic acid, and methionine, in different compositions and proportions depending on the operation conditions (Figure 10).

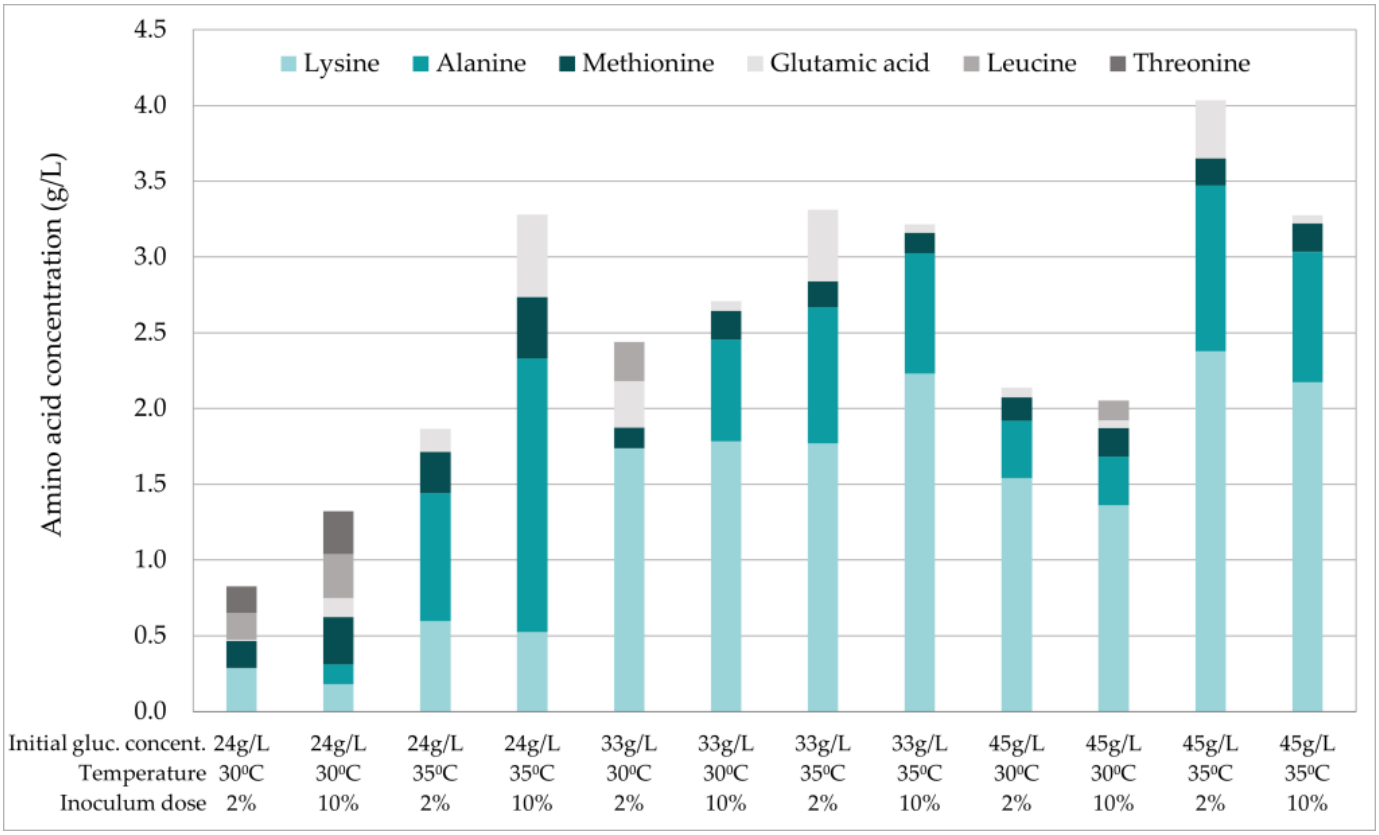


Figure 10. Amino acid concentration after 72 h fermentation of different glucose concentration hydrolizates by *C. glutamicum* ATCC21492 at the studied conditions.

3.6. SSF Performance

In this section, results of L-lysine and other amino acids production, as well as residual glucose after 72 h of SSF by Fiberlife® 550 enzymatic blend and *C. Glutamicum* under various experimental conditions are shown.

Figure 11 shows L-lysine production in all SSF assays. Remarkably, L-lysine titers are much lower than those obtained with SHF. Previous studies on enzymatic hydrolysis suggested that the presence of certain nutrients in the fermentation medium could negatively affect hydrolysis efficiency. This factor may explain the low glucose release, leading to the low L-Lysine titers across the different setups.

In addition, Figure 12 presents the L-lysine yield expressed per gram of solid substrate (cotton), providing an alternative perspective on production efficiency.

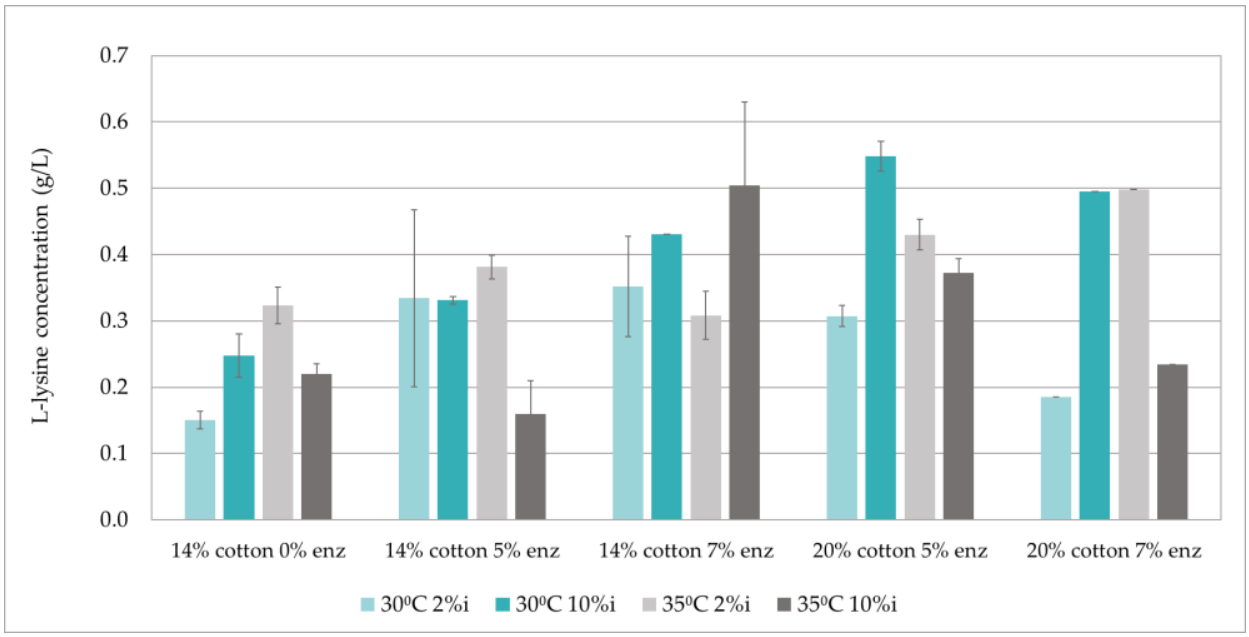


Figure 11. L-lysine concentration after 72 h Simultaneous Saccharification and Fermentation (SSF) by *C. Glutamicum* ATCC21492 at the studied conditions.

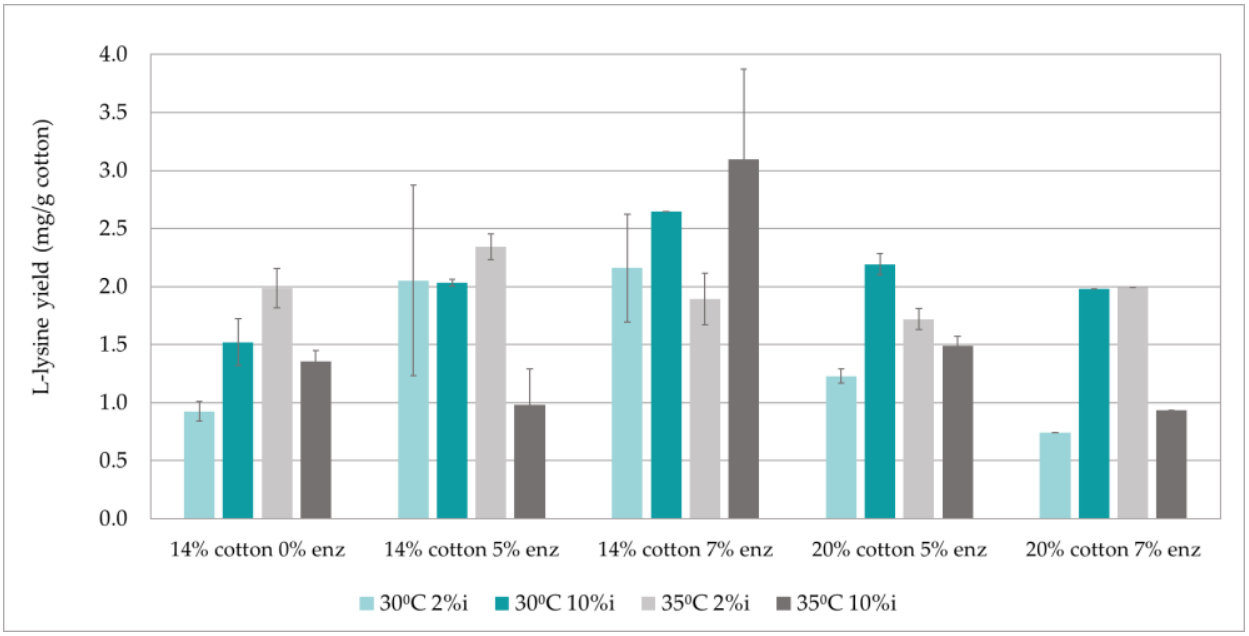


Figure 12. L-lysine yields after 72 h SSF by *C. Glutamicum* ATCC21492 at the studied conditions.

Figures 11 and 12 show the concentration and yield of L-lysine under various SSF conditions. The highest L-lysine concentration was obtained with 20% cotton, 5% enzyme dose, at 30 °C, and 10% inoculum. Other favorable conditions included 14% cotton, 7% enzyme dose, at 35 °C and 10% inoculum; 20% cotton, 7% enzyme dose, at 30 °C and 10% inoculum; and 20% cotton, 7% enzyme dose, at 35 °C and 2% inoculum. These conditions generally combined two or three parameters at their highest tested levels, consistent with observations in SHF experiments.

However, when results are expressed as L-lysine yield per gram of cotton (Figure 12), a different trend is observed. Although 20% cotton led to the highest L-lysine concentrations, the yield was actually lower compared to fermentations using 14% cotton. This is due to a

lower liquid-to-solid ratio at higher solid loadings, which may have limited mass transfer and nutrient availability, a phenomenon also observed in studies using other substrates for L-lysine production [26].

Additionally, an increasing inoculum dose tended to enhance L-lysine production at 30 °C, while at 35 °C, higher inoculum doses resulted in reduced productivity. This suggests a temperature-dependent interaction between biomass concentration and metabolite synthesis.

It can be highlighted that in the absence of enzymes, L-lysine production was also observed, indicating that *C. Glutamicum* found another carbon source, probably coming from the components present in the culture medium. Given the basal medium composition, casein emerges as a plausible candidate, as this bacterium is unable to degrade cellulose.

Regarding glucose dynamics, Figure 13 shows the residual glucose concentrations after 72 h of SSF. This parameter encompasses both the enzymatic release of glucose from cotton and its subsequent consumption by *C. glutamicum* as a carbon source. An increase in both enzyme load and substrate concentration promoted glucose release, with the highest residual glucose levels observed at 20% cotton and 7% enzyme. However, residual glucose concentration varied according to temperature and inoculum level.

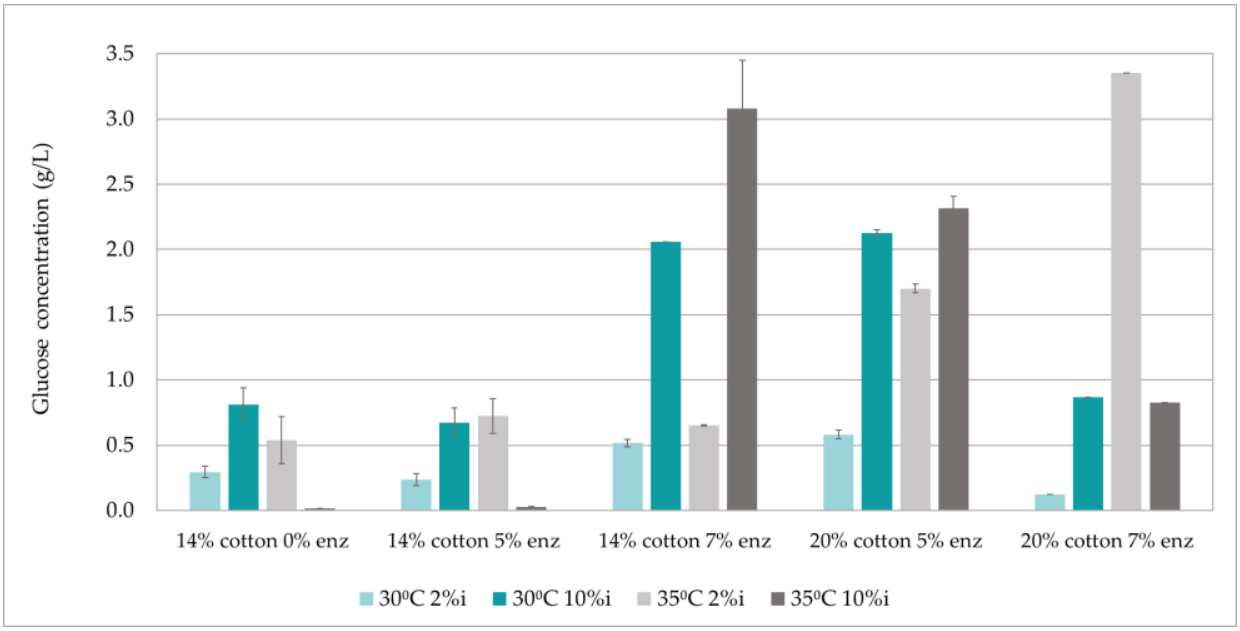


Figure 13. Residual glucose after 72 h SSF by *C. Glutamicum* ATCC21492 at the studied conditions.

On the consumption of released glucose, typically, elevated residual glucose levels are interpreted as indicators of metabolic inhibition. However, in the present study, the highest concentrations of residual glucose coincided with increased production of L-lysine and other amino acids. These findings highlight the complexity of substrate utilization dynamics during fermentation.

Finally, Figure 14 shows the concentration of amino acids obtained after the different SSF cotton tests for 72 h. It is noteworthy that the composition is different from that obtained with SHF, especially in relation to the concentrations of threonine and leucine, which are predominant over lysine and alanine, which were the most abundant amino acids present in the SHF tests.

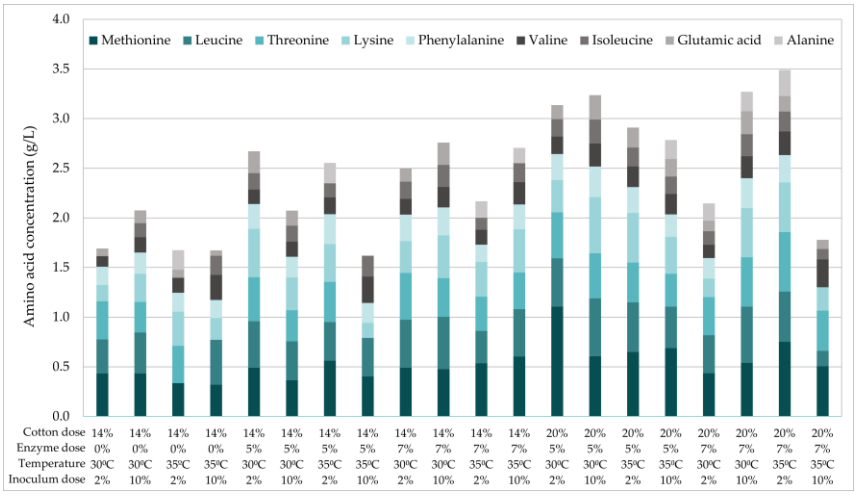


Figure 14. Amino acid concentration after 72 h SSF by *C. Glutamicum* ATCC21492 at the studied conditions.

4. Discussion

4.1. Interpretation of C. glutamicum Growth Patterns

The growth of *C. glutamicum* ATCC 21492 was evaluated using glucose as the sole carbon source. As previously mentioned, the lag phase lasted approximately 6 h, followed by the exponential growth phase extending to 24 h. Nevertheless, the bacterial growth continued to rise, peaking at 26 h with a concentration of 2.73×10^9 CFU/mL (Figure 4). The microorganism may have entered the stationary phase around 24 h. However, the standard deviations at the 24 and 30 h time points are relatively high, suggesting that the bacterial population may be in a transitional phase between exponential growth and the stationary phase. These findings are consistent with previous reports that document comparable lag phases under glucose-rich culture conditions [27].

The maximum specific growth rate obtained in this study (0.8317 h^{-1}) is comparable to other studies. For example, Bashir et al. (2022) [28] reported a specific growth rate of 0.519 h^{-1} for *C. glutamicum* cultivated in media containing 50 g/L glucose at 30 °C, and a shaking speed of 120 rpm, conditions closely aligned with those used in the present work.

Among the kinetic models assessed, the modified Gompertz model exhibited a good determination coefficient (R^2, 0.95). Better adjustment may be obtained by reducing the large variation among the replications, which could be caused by different physiological factors mentioned below. An interesting result was obtained by using the beta model, which provided an R^2 equal to 0.97. However, signs of overfitting were observed, likely due to sensitivity to outlier data points. In this way, due to its robustness and better parameter interpretability, the modified Gompertz model appears to be the most appropriate for describing the observed growth profile. Therefore, although other models can be used, the modified Gompertz model better represents the growth of the *C. glutamicum* under the operating conditions tested in the present study, in agreement with existing literature that supports the suitability of this equation for modeling microbial growth [28]. Several environmental and physiological factors are known to influence the growth dynamics of *C. glutamicum*, including oxygen availability, substrate concentration, and the accumulation of metabolic by-products. In particular, oxygen limitation has been shown to restrict respiratory activity, thereby reducing growth rates and the biosynthesis of industrially relevant metabolites [29]. This parameter could not be controlled as the bacterial growth was performed in flasks, which is likely responsible for the variation among replications. In addition, the buildup of organic acids and other by-products can perturb central carbon

metabolism, diminishing substrate utilization efficiency and expediting the onset of the stationary phase [25].

In the current study, the transition into the stationary phase was observed to begin around 24 h, which aligns with previous observations indicating that oxygen limitation and metabolic by-product accumulation serve as key triggers for growth arrest in *C. glutamicum* [27,28]. These insights underscore the importance of implementing optimized aeration strategies in future cultivation experiments to extend the exponential phase and enhance biomass yield.

In summary, the growth behavior of *C. glutamicum* in glucose-containing media observed in this study is consistent with established trends reported in the literature. Future research should focus on strategies such as improved oxygenation to further enhance growth efficiency and broaden the applicability of *C. glutamicum* in industrial bioprocessing.

4.2. Analysis of SHF Efficiency and Process Variables

The experimental results confirm that initial glucose concentration plays a key role in modulating L-lysine production by *Corynebacterium glutamicum* ATCC 21492. As glucose levels increased from 24 to 33 and 45 g/L, L-lysine titers also improved. However, the marginal gain observed at 45 g/L was less pronounced, suggesting the presence of a saturation threshold beyond which additional carbon does not lead to proportional increases in L-lysine synthesis. This trend is consistent with prior studies and also with the fermentation assays carried out using commercial glucose, reporting metabolic limitations under high-substrate conditions [13].

Temperature was another critical parameter. Cultivations at 35 °C yielded higher L-lysine concentrations compared to 30 °C, particularly at elevated glucose levels. Nevertheless, the modest enhancement at 35 °C may reflect early signs of thermal stress, as enzyme and metabolic activities in *C. glutamicum* are known to decline above 33 °C [17,23]. These findings align with reports defining the optimal temperature window between 30–35 °C, depending on the strain and medium composition.

The influence of inoculum size on process performance underscores its role in modulating metabolic activity. In particular, higher inoculum sizes enhanced L-lysine titers at 30 °C, while at 35 °C, increased inoculum sizes led to reduced production. This suggests a need for further investigation into the relationship between inoculum size and metabolic efficiency under varying temperature regimes.

As previously discussed, this variability could be attributed to minor variations in the timing of preculture preparation. Since the precultures were collected at 24 h, coinciding with the exponential growth phase of *C. glutamicum*, even slight differences in preparation time could lead to significant changes in the initial bacterial population density.

Residual glucose concentration patterns suggest that substrate utilization is closely linked to both temperature and inoculum levels. The reduction in glucose uptake at higher concentrations may point to limitations in transport mechanisms or the accumulation of inhibitory by-products, emphasizing the importance of balancing substrate availability with metabolic capacity to optimize L-lysine synthesis.

These observations illustrate the intricate, non-linear interactions between process variables such as temperature, inoculum size, and substrate concentration, which collectively shape metabolic outcomes.

Further metabolite analysis of the fermentation broth revealed the presence of additional amino acids, suggesting that *C. glutamicum* redirected part of its metabolic activity in response to the culture conditions. This behavior aligns with the known metabolic versatility of *C. glutamicum*, which can synthesize a range of amino acids depending on

the environmental and nutritional context [10,17]. In particular, glutamic acid is commonly excreted due to the accumulation of α-ketoglutarate from the TCA cycle, especially under stress or excess carbon flux [19]. L-lysine and threonine are derived from the aspartate pathway and are favored by increased anabolic flux under high biomass conditions [20], while alanine, synthesized from pyruvate, acts as a metabolic overflow route that helps balance redox and relieve carbon excess during high glycolytic activity [21]. These findings highlight the organism's ability to dynamically reroute metabolic fluxes and underscore the importance of fine-tuning fermentation conditions to improve the selectivity of product formation.

Compared to literature benchmarks, the L-lysine titers achieved in this study were modest (2.38 g Lysine/L and 52.88 mg lysine/g glucose or 2.23 g lysine/L and 67.59 mg lysine/g glucose using 45 g glucose/L hydrolysate and 2% inoculum size or 33 g glucose/L hydrolysate and 10% inoculum size respectively, both at 35 °C). Xu et al. (2019) [13] reported titers up to 121.4 g/L (460 mg lysine/g glucose) using fed-batch fermentation, 10% inoculum size at 30 °C with metabolic engineering approaches targeting redox balance, such as GAPDH and IDH modification. Other studies emphasized minimizing flux diversion toward by-products. Mimitsuka et al. (2007) [24] demonstrated the need to regulate cadaverine synthesis, yielding 5.2 g/L L-lysine (104 mg lysine/g glucose) in 18 h at 30 °C, while Hussain et al. (2015) [22] highlighted how media optimization can significantly boost L-lysine production (up to 3.5 g/L, or 70 mg lysine/g glucose in 72 h at 30 °C).

Although glucose was used as the primary carbon source in this study, it is important to highlight that the casein added to the basal fermentation medium can also act as an additional carbon source. This supplementation may enhance the productivity of specific metabolic pathways. Similar findings have been reported in previous studies, such as Alcántar-Garduño et al. (2000) [30], who increased L-lysine production to 595 mg/L within 48 h under optimized culture conditions.

These insights indicate that the baseline results reported here represent a solid foundation for further improvement through integrated strategies involving strain engineering and culture medium design.

An additional strategy to optimize fermentation for L-lysine production with *C. glutamicum* involves oxygenation control, which was not addressed in this study but is proposed for future research. Recent studies highlight that efficient oxygen transfer can significantly enhance L-lysine yields and productivity. For instance, advanced bioreactor designs with improved oxygenation systems have achieved L-lysine concentrations of up to 208.36 g/L and glucose-to-lysine conversion rates of 83.3%. These findings emphasize the critical role of oxygenation in optimizing fermentative amino acid production processes [31].

4.3. Analysis of SSF Efficiency and Process Variables

L-lysine production was consistently lower in SSF assays compared to SHF experiments. One possible explanation is that the basal medium components during enzymatic hydrolysis may have negatively affected enzyme activity, leading to a low glucose release and thus a low L-Lysine titer across the different setups. It was actually observed in enzymatic hydrolysis assays using culture broth as a liquid medium. Furthermore, high substrate loads resulted in poor hydration, limiting mass transfer, which can impede enzymatic hydrolysis efficiency and microbial metabolism. Studies have shown that at solid loadings above 15%, the hydrolysis of lignocellulosic materials is significantly hindered by mass transfer constraints and reduced mixing efficiency [32–36].

Interestingly, this temperature-dependent behavior of inoculum size on L-lysine production mirrors the trends observed in the SHF experiments using commercial glucose as

the carbon source. This consistency across both SHF and SSF processes underscores the significant influence of temperature on the relationship between biomass concentration and metabolic output in *C. glutamicum* fermentations.

On the other hand, unexpected metabolic activity was also detected in SSF control assays without enzyme addition, where only cellulose was present as a carbon source, suggesting that *C. glutamicum* utilized alternative substrates, such as casein, also a nitrogen source. This hypothesis is further supported by differences in amino acid profiles between the two processes. While SHF assays predominantly produced L-lysine, followed by lower amounts of alanine, SSF assays exhibited a shift in amino acid composition, with methionine and leucine as the most abundant products, followed by threonine and L-lysine in fourth position. These results underscore the critical influence of medium composition and enzymatic efficiency on product yield and metabolic pathways under SSF conditions.

The observation that the highest residual glucose concentrations coincided with increased L-lysine and other amino acid production suggests that the slow kinetics of cotton hydrolysis led to delayed glucose availability. Consequently, during the initial stages of fermentation, *C. glutamicum* likely utilized casein as an alternative carbon source. As glucose became more abundant over time, it supplemented the metabolic processes, contributing to enhanced amino acid synthesis.

Comparatively, SSF has been successfully applied to L-lysine production in other studies. For example, Egbune et al. (2024) [26] reported 3.27 mg of L-lysine per gram of dry substrate using *C. glutamicum* ATCC 13032 and peanut cake as feedstock, higher than mg/g cotton While process parameters and substrates vary, both studies highlight the feasibility of SSF for L-lysine biosynthesis and the importance of optimizing solid load, inoculum size, and temperature to enhance process performance.

These findings further illustrate *C. glutamicum*'s capacity to adapt its metabolic pathways in response to varying fermentation conditions, emphasizing the critical role of optimizing process parameters to enhance L-lysine production.

5. Conclusions

This study demonstrates the potential of utilizing cotton-derived glucose hydrolysates as a carbon source for L-lysine production by *Corynebacterium glutamicum*. Optimal L-lysine concentrations were achieved with initial glucose concentrations between 30 and 50 g/L. However, the interplay between fermentation temperature and inoculum size revealed a complex relationship affecting metabolic efficiency; notably, simultaneous increases in both parameters led to diminished yields.

Beyond L-lysine, the fermentation process also resulted in the production of other amino acids, including alanine, threonine, methionine, and leucine. The concentrations and proportions of these amino acids were influenced by the specific operational conditions, highlighting the organism's metabolic flexibility.

In Simultaneous Saccharification and Fermentation (SSF) assays, the enzymatic hydrolysis step, particularly when employing Fiberlife® 550, emerged as a limiting factor. This limitation likely prompted *C. glutamicum* to utilize alternative carbon sources, such as casein present in the medium, leading to a metabolic shift favoring the production of methionine, leucine, and threonine, with L-lysine being produced in lesser amounts. Despite this shift, the total concentration of amino acids produced in SSF was comparable to that achieved using glucose as the sole carbon source.

These findings underscore the importance of optimizing fermentation conditions and enzyme efficiency to enhance L-lysine production. Future research should focus on improving the saccharification process and further elucidating the metabolic pathways involved in amino acid synthesis under varying fermentation conditions.

To contextualize the feasibility of L-lysine production at scale, we performed a Fermi estimation based on the availability of cotton residues. Assuming an approximate global annual cotton fiber production of 26 million tons [37] and estimating that cotton waste accounts for around 10–20% of this value, this translates to 2.6 to 5.2 million tons of potential feedstock. Applying our experimental yield of 22.30 kg of L-lysine per ton of cotton, the theoretical annual global L-lysine production from cotton residues could range between 58,000 and 116,000 tons. Further refinement of enzymatic hydrolysis and fermentation parameters may unlock even greater production potential.

Author Contributions: Conceptualization, D.F.G.; Methodology, A.G.A., A.O.B. and D.F.G.; Investigation, P.R.B., A.G.A. and D.F.G.; Data curation, A.O.B.; Writing—original draft, P.R.B. and A.G.A.; Writing—review & editing, P.R.B., A.G.A. and D.F.G.; Supervision, A.G.A. and D.F.G. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

ADF	Acid Detergent Fiber
ADL	Acid Detergent Lignin
CFU	Colony-Forming Units
EH	Enzymatic Hydrolysis
HPLC	High-Performance Liquid Chromatography
NDF	Neutral Detergent Fiber
PAD	Pulsed Amperometric Detector
RMSE	Root Mean Square Error
SHF	Sequential Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation
TSB	Tryptic Soy Broth (TSB)
UNEP	United Nation Environment Program

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