Journal of Engineering Science and Technology
Vol. 17, No. 1 (2022) 0673 - 0688

SYSTEMATIC COMPARISON OF GLUCONIC ACID OPTIMIZATION PRODUCED BY *ASPERGILLUS* USING RAW BIORESOURCES CULTIVATION MEDIUM

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Abstract

Several researchers discussed the influence of fungus and medium components on the gluconic acid quantity and quality. The new trend of research is focusing on finding cheaper alternatives for the carbon source used in the medium. Scientists cannot achieve this goal until the compatibility of medium and fungus is investigated. Thus, the current study aims to compare gluconic acid production by *A.niger, A.fisheri and A.carneus* systematically. Also, it examines the possible optimization of cultivation medium by using corn starch, banana must and molasses as cheap carbon source. The key parameters to identify the successful optimization are gluconic acid production, cell dry weight, biomass content and total reducing sugar. The result of the study showed that *A. niger* has 10.4 g/L gluconic acid at medium 2 which contains glucose. A pivotal proportional relation between the raw carbon source concentration and acid production was revealed. The most intriguing finding is the drastic reduction in the sugar content between 50% to 80%. Moreover, the molasses showed an additional 20% enhancement of gluconic acid by *A.niger* in the absence of glucose. The molasses outstanding performance at the end of the bioreactor stage showed a competitive and possible alternative to glucose at the industrial level.

Keywords*: A. niger*, Carbon sources, Gluconic acid, Molasses, Optimization media.

1. Introduction

Gluconic acid is an important organic acid that could be obtained from the Glucose oxidation [1]. The unparalleled characteristic of the acid such as: low toxicity, low corrosivity and metal tolerance ability, broaden the acid implementation. Accordingly, the implementation has been extended to various areas such as: pharmaceuticals, food, textile, metal chelating agent production, leather, beverages and dairy industries. Gluconic acid has witnessed excessive demand growth over the last decade, especially in pharmaceutical and modern biological applications [2]. The global market reached nearly 800,000 tons annually [3].

At the industry level, gluconic acid was mainly produced by four main processes. The first process is the chemical process through oxidizing the glucose using hypochlorite solution [4]. The second process is the fermentation process by microorganisms. The fermentation process depends on implementing the appropriate culture medium, which usually contains glucose and other components [5]. Thirdly, using electrolytic glucose oxidation of the solution in the presence of bromide was famous too [6]. Fourthly, using the photocatalytic process such as catalysing water by an effective synergistic photocatalytic system [7]. Until now, these techniques were applied at different magnitudes.

Gluconic acid production by fermentation has been an interesting subject for researchers [8]. The fermentation process has been confirmed as an effective and controlling technique for gluconic acid manufacturing [9]. The fermentation / microbial method adaptation reduced the technical problems associated with chemical methods. For instance, the fermentation overcame the drawbacks of chemical side reactions and promoted the yield increase and acid production quality [10].

In the fermentation process, many new species of microorganisms became under focus. *Aspergillus* [11] *Penicillium* [12] *Pseudomonas fluorescens* [13] *Pseudomonas plecoglossicida* [14] *Arthrobacter globiformis* [15] *Gluconobacter* [16] and *Zymomonas* [17] for gluconic acid production. Many researchers found *Aspergillus* as the breakthrough in producing gluconic acid at the industrial level. *Aspergillus* is genus of fungi that consists of approximately 300 mold species. It can be found in various environments throughout the world. They commonly exist in nature, with their spores being abundant in the air. The characteristic of *Aspergillus* is significant to be studied. For instance, studies have indicated that *Aspergillus* can tolerate extreme conditions only if all other conditions were ideal [18, 19]. Noting that *Aspergillus* growth depends on water availability as well as temperature. Also, since *Aspergillus* have different breeds, selecting among them for a specific property is achievable. Considering *Aspergillus* for gluconic acid production resulted from the ability of *Aspergillus* to produce all the enzymes desired for the conversion of glucose into gluconic acid. The enzymes contain catalase, glucose oxidase, mutarotase and lactonase. Usually, crystalline glucose monohydrate comes at the alpha form and is converted to beta form during gluconic acid production [1, 20]. Another fact that assists *Aspergillus* is the production of mutarotase, which plays a crucial role as a reaction accelerator during the glucose conversion to gluconic acid [1, 21].

Due to environmental, process mechanism and availability, comparing different *Aspergillus* geners is crucial [22]. Therefore, research trends have focused on genre enhancement producers [22]. At the same time, other studies have explored improving and controlling the *Aspergillus* morphology and improving the gluconic acid quality [19]. Despite the different attempts to boost gluconic acid production by *Aspergillus*, it is not yet economically feasible.

It is known that the fermentation process requires a substantial quantity of sugar, which is the primary carbon source. The traditional sources as glucose, sucrose, and lactose were the most common. Until recently, there has been no reliable evidence that using cheap raw carbon materials to minimize the fermentation process's expenses could entirely replace the traditional carbon sources. Some investigations reported that the cost of glucose was around 20-30 times higher than molasses. This comparison's common factor is the chemical composition- since molasses is composed of glucose [23, 24].

Moreover, the raw cheap carbon sources were mainly investigated as additives to the traditional carbon sources. For example, Corn stover as a source of lignocellulosic biomass was studied to improve the fermentation process of *A. niger* [25]. The major finding reported is that gluconic acid production was 76.67 g/L and a yield of 94.91% without any inhibitors. Also, fig was investigated as a medium to produce gluconic acid and citric acid via solid-state fermentation. The results of the study revealed a great production of gluconic acid over time [26]. Grape-must was used as a cheap carbohydrate source to produce gluconic acid. gluconic acid yield (%) was 70:5 at 24 h of the process. The study confirmed that grape-must was a beneficial carbon source for gluconic acid fermentation [27]. An early attempt to use cheese whey for fermentative production was coupled with 0.5% of glucose and immobilized mycelia. 33% additional gluconic acid was obtained by the method [28]. A comparative study of the wastepaper hydrolysate to glucose showed high production for both flask and bioreactor [29]. Interestingly, a merge between the hydrothermal method and potato pulp for fermentation was studied. The hydrothermal as a pre-treating method advanced gluconic production. Each 1000 gm. of potatoes produced 546.48 gm of gluconic acid [30]. Cassava showed significant improvements in cultivation process parameters in 16-L stirred tank bioreactor [31]. Although some attempts have been made to address the optimization possibility economically, there is still a need for critical evaluation before implementation. Thus, carrying systematic research on different *Aspergillus genera* is required.

In general, much research has been done on *Aspergillus* genera and specifically on *A. niger*. Nevertheless, other species were not thoroughly investigated. Thus, the present study is a three-stage examination for gluconic acid production by *A. niger, A. fisheri and A. carneus*. The second stage investigates the possible medium optimization by three cheap feedstocks: Corn starch, Molasses and Banana Must. The second stage is considered as a benchmark for cheap materials replacing the traditional carbon source. Therefore, the decision on the successful candidates depends on gluconic acid productivity. The analysis adopted one factor at time (OFAT) strategy to profit the most appropriate medium composition for improving gluconic acid production. Finally, fermentation was scaled up by a bioreactor of 4-L scale.

The study is one step toward industrializing the cheap sources as an alternative for gluconic acid production.

2. Methodology

For our current study approach, it was conducted at several stages. The tasks vary at each stage, while the leading indicator is the gluconic acid remained the utmost goal. Figure 1 depicts the flow chart of the operation. The study details are provided in the following sections.

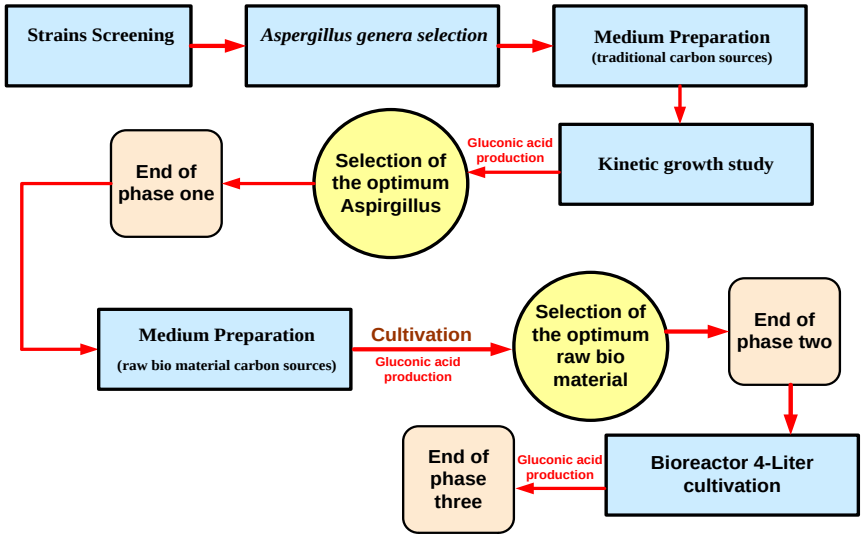

Fig. 1. Flow chart for gluconic acid production from bioresources materials.

2.1. Microorganisms

A. niger, *A. fisheri* and *A. carneus* isolated and preserved in ULT deep freezer at a temperature of -80°C. The fungus was received in a frozen glycerol solution. Before sterilization, the medium acidity was tuned to neutral acidity (pH 7.0). Sequentially, a sufficient time of 96 h at a controlled temperature of 30°C was attained for the inoculated flasks incubation. After the colonies arose, the collection was made by using glycerol stocks at 50% concentration. Finally, all the tubes were stored at -80°C in ULT deep freezer and practically considered as working cell bank**.**

2.2. Fermentation medium

For gluconic acid medium optimization experiments, (*A. niger*, *A. fisheri* and *A. carneus*) were cultivated from different media. The composition of the three mediums under investigation is illustrated in Table 1. Erlenmeyer flasks at a volume of 250 mL were used limitedly for a working volume of 50 mL, which helped the fungus cultivations. It is worth noting that medium 1, 2, 3 were pH neutralized by using HCL at room temperature 25 °C.

Table 1. Media components.

Media	Components .Formula g/L
Medium 1	Sucrose 30.0, $NaNO_3$ 3.0, KH_2PO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, KCl 0.5, $FeSO_4{\cdot}7H_2O$ 0.01.
Medium 2	Glucose 50.0, $NaNO_3$ 3.0, yeast extract 3.0, KH_2PO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, KCl 0.5, $FeSO_4{\cdot}7H_2O$ 0.01.
Medium 3	Glucose 50.0, Peptone 2.0, KH_2PO_4 2.0, $MgSO_4{\cdot}7H_2O$ 0.5**.**

2.3. Gluconic acid preparation

The study includes two main stages, which are the flask scale stage and the bioreactor stage. Accordingly, to prepare the gluconic acid samples after the medium cultivation step, two flask sizes were used. The flask size of 50 ml was used for shaking purposes to allow enough volume for homogeneous shaking. The 20 ml flask was used in broth bioreactor experiments. The study's key parameters were the effect of pH, dissolved oxygen DO, Cell dry weight, Total reducing sugar and gluconic acid concentration. 4-L bioreactor (LH 500 series; LH Fermentation Ltd., Bells Hills, U. K) was used at the bioreactor stage. The samples were centrifuged immediately at 5 ºC to separate cells from the fermentation broth. The supernatants were stored immediately at -20 ºC for Gluconic acid analysis.

2.4. Gluconic acid characterization

The gluconic acid extraction process for cells was obtained by a high-speed centrifuging of 6000 rpm for 10 minutes at 4 ºC. The high-performance liquid chromatography (HPLC) used for concentration measurement consisted of Waters 2690 Alliance Separations Module with Waters. 996 Photodiode Array Detector, Column": Hi-Plex H, 300 × 7.7 mm with guard column). For separation, the flow rate was adjusted at 0.6 mL/min at a temperature of 40°C. Since the acid is a short organic chain, the mobile phase for the detection used was 0.005N H_2SO_4. The HPLC was equipped with Ultraviolet detection and was used at 210 nm [32].

2.5. Cell dry weight measurement

For the dry weight, the precipitate was washed three times by distilled water. The cleaned samples were placed in a centrifuge to separate any water. The collected sample was placed in the oven and dried at 80 ºC .

2.6. Determination of total reducing sugar

Total reducing sugar content was estimated according to [33]. using the dinitro salicylic acid (DNS) method.

2.7. pH determination

pH is a very significant bioprocess parameter in the fermentation process in the study using pH Meter model (Mettler Toledo Delta 320).

2.8. Optimization of gluconic acid Production using bioresources

In order to optimize gluconic acid production, three main bioresources were systematically evaluated. Corn starch, Banana must, and molasses were assessed based on the optimum concentration required to maximize the gluconic acid production.

For corn starch, the feedstock was prepared by wet milling procedure at the laboratory. The corn immersed in 0.50% (w/v) lactic acid and 0.15% (w/v) sodium bisulphite. It was left for 48 h at 50 ± 1°C, and the procedure was found useful in the literature [34]. For the banana must, the fruit obtained from a local market. During the banana selection, we selected the damaged fruit since it was not edible. Banana juice was prepared and heated. Also the molasses obtained from a local market and storage at - 4º C.

In order to provide credibility to the comparison, all the biosources concentration used was uniform. This allowed the critical result judgment between the samples. The concentration range used for the evaluation was 0, 10, 20, 30, 40 and 50 g/L. The values selected were found during the preliminary screening and validated by one factor at a time (OFAT) strategy [35].

3. Results and Discussion

3.1. Gluconic acid at different media

Gluconic acid detection via High-performance liquid chromatography (HPLC) by the comparison of the retention time. The selected *Aspergillus* were able to produce gluconic acid with different properties, as seen in Figs. 2-4.

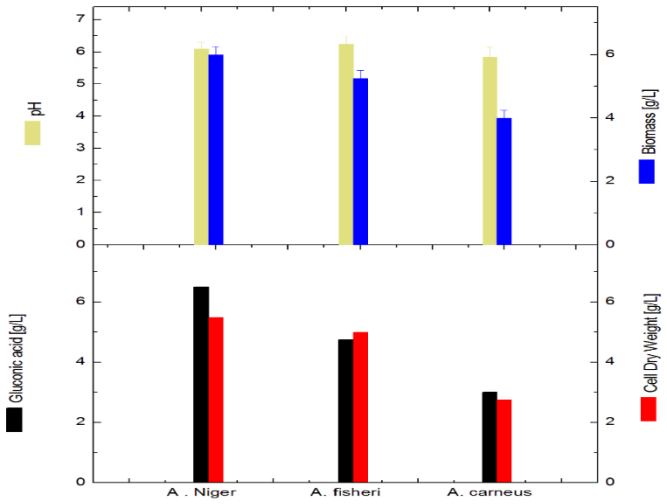

Fig. 2. Gluconic acid production properties using medium 1.

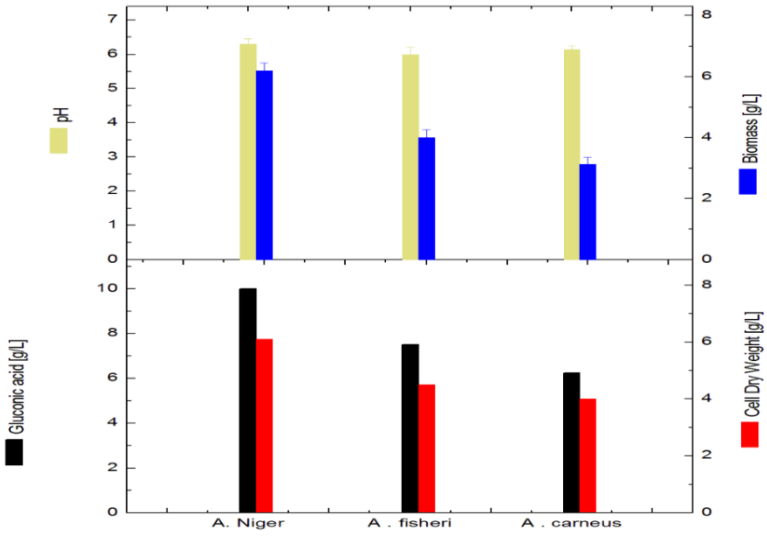

Fig. 3. Gluconic acid production properties using medium 2.

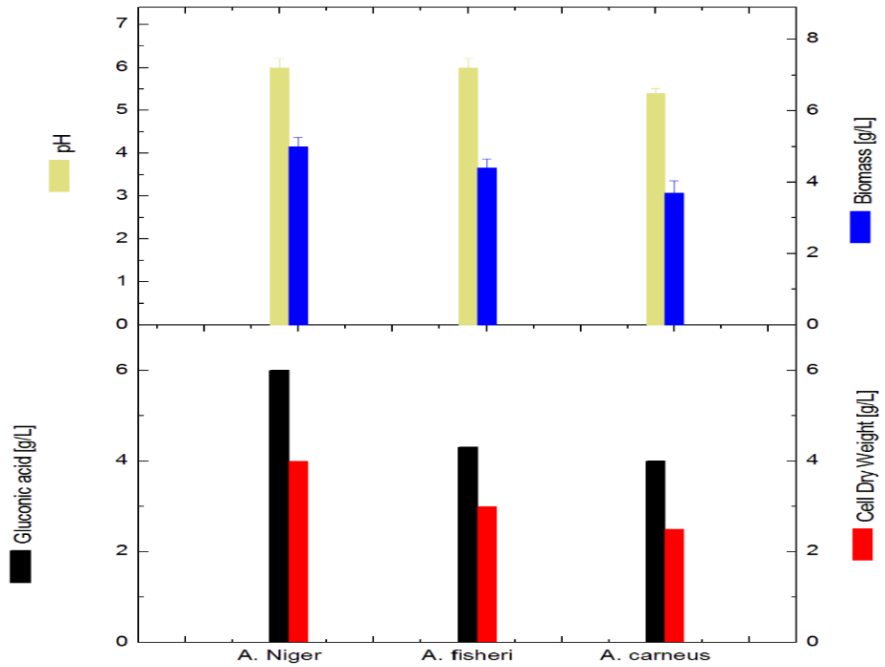

Fig. 4. Gluconic acid Production Properties using medium 3.

By looking at the results of *A. niger* the gluconic acid production was the highest at medium: 2. The results in medium 1 and 3 were similar, and no significant variation was noticed. The gluconic acid showed an additional 4 g/L, which is 66% in comparison to medium 1 and 3.

Regarding the cell dry weight and biomass quantity *of A. niger* , it is clear that the minimum quantities were obtained is in medium: 3. While the highest was in medium 1. Related results were found by recently conducted similar researches [27, 36]. However, the current study showed significantly higher gluconic acid production between 17%-25%.

The change in *A. fisheri* ability to produce gluconic acid is noticeable. Figure 3 shows that the highest gluconic production was in medium 2, considered a similar trend found for the *A. niger*. The gluconic production is 3.5 g/l more than the average of medium 1 and 3, which is equivalent to 66% additional production. The dry weight of gluconic acid was at highest in medium 1 with around 1.5 g/L additional quantity.

The same trend of dry weight was noticed for biomass quantity in *A. fisheri*, Because the source of carbon in medium 1 was sucrose, but in medium 2 was glucose. The capability of *A. fisheri* ability to produce gluconic acid in the presence of glucose as carbon source proved beneficial and was endorsed in [37].

Focusing on *A. carneus*, the gluconic acid production, was highest in medium 2 by around 2.25 g/L from the average of medium 1 and 2. Also reflected 79% additional gluconic production. The cell dry weight and the biomass quantities were surprisingly different compared to the other two genera. The cell dry weight quantity was the highest in medium 2, while the biomass was the highest in medium

1. The source of carbon in medium 2 was glucose, but in medium 1 was sucrose. So due to the different carbon sources, the trend likely to be altered. Pinpointing that the trend is specifically opposite to *A. fisheri*, according to [38]. The superiority of glucose as a carbon source assisted in gluconic acid production.

As for the pH value is one of the most critical factors affecting on the fungal growth as well as the formation of organic acids. The profiles gluconic acid with respect to initial pH of the fermentation medium is shown in Figs. 2-4. Over 50% yield of gluconic acid was observed in a pH range from 6 to 6.5. The best production was 10.00 g/l by *A. niger* at pH 6.5. The appropriateness of pH ranges 5-6 for both the growth and gluconic acid production in all three media. And this completely correspond with literature in [27, 39, 40].

Based on the available data, the highest gluconic acid produced was as follows: *A.* niger, *A. fisheri and A. carneus*, respectively, For the cell dry weight, the highest was obtained by *A. niger*. According to the analysis, *A. niger* demonstrated superior performance and greater dry cell weight. Thus, *A. niger* is the best genera for gluconic acid production, specifically at medium 2.

3.2. Optimization gluconic acid production by bioresources

As the optimization of gluconic acid production is considered a crucial step towards production sustainability, cheap mediums were used for the purpose. Corn starch, banana must, and molasses considered inexpensive carbon sources. The study of the bioprocess parameters such as growth kinetics of cell growth, Carbon source concentration, pH and temperature cultivation were compared.

3.2.1. Corn starch for gluconic acid production optimization

Figure 5 shows that gluconic acid production increased with the increase in corn starch concentration. The maximum gluconic acid of 12.00 g/L was acquired at 96 h fermentation at the 50 g/L of corn starch concentration, which indicates addition of 2.00 g/L to the production. Moreover, it was noticeable a slight incremental addition in gluconic acid production between the concentrations of 30 g/L and 40 g/L. The trend of gluconic acid increasing is proportional to the concentration of the corn starch but not linear.

Additionally, the presence of 50 g/L of corn starch resulted in cell dry weight and biomass recorded 7.50 g/L, 11.00 g/L, respectively.

These results showed the ability of corn starch to assist gluconic acid production. Furthermore, corn starch assisted in reducing the sugar content to 0.50 g/L. This reduction of sugar content is promising since the enhancement is estimated by 90%. These findings align with those found in gluconic acid study's by using some microorganisms [41]. It is also indicated that gluconic acid production has 14% additional to what has been previously reported. Nevertheless, the production values are assumed marginal at most.

3.2.2. Banana must for gluconic acid production optimization

By using banana must at 50 g/L for optimization, the gluconic acid has production around 6 g/L. Like corn starch, the gluconic acid is in proportional relation to the concentration, as seen in Fig. 6.

However, gluconic acid production is insensitive to the banana must concentration between 30-40 g/L concentration. Despite the production trend, it was important to highlight the effect of banana must on cell dry weight and biomass. The cell dry weight and biomass is 7.00 g/L and 6.50 g/L, respectively.

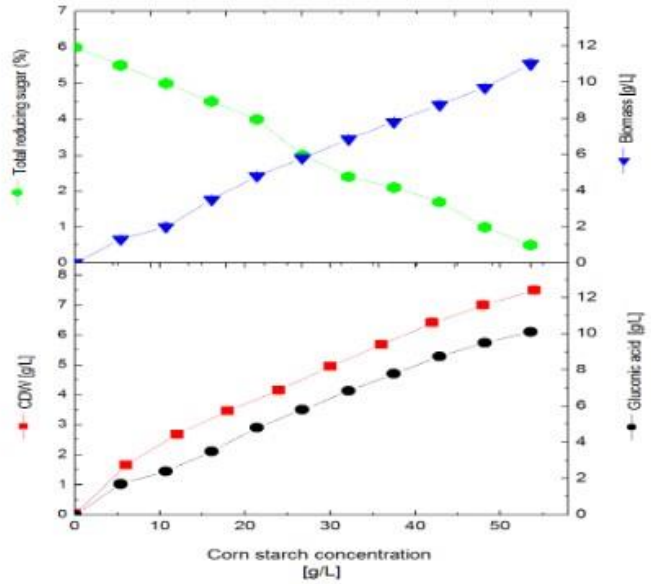

Fig. 5. Gluconic acid production after cultivation in the optimization medium contains various concentrations of corn starch at 30°C for 96 h.

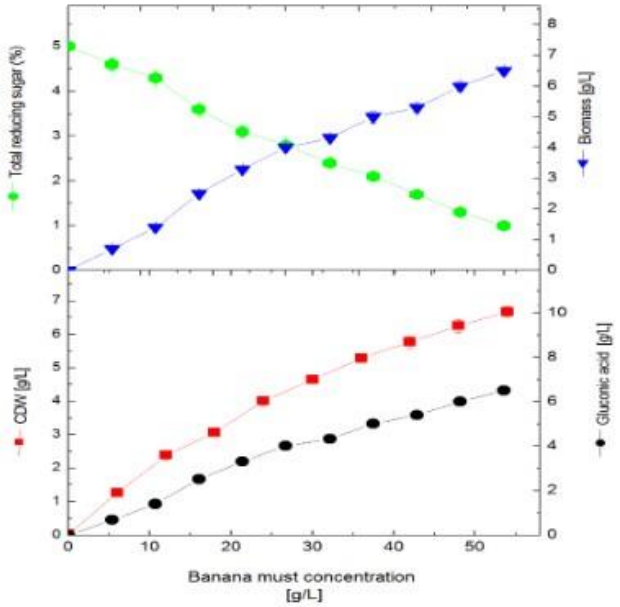

Fig. 6. Gluconic acid production after cultivation in the optimization medium contains various concentrations of banana must at 30°C for 96 h.

This significantly contributed to the source potentiality. From the observations, it is noticed that the total reduction in sugar content is inversely proportional to the increased concentration of banana must. The reduction estimated by 80%, which is lower by 10% compared to the corn starch samples. It worth referring that the current study followed a similar trend to gluconic acid Production by *A. niger* from banana must but at different bioprocess parameters [42].

Moreover, they reported only 4.14 g/100 mL , which is approximately lower the current research findings. The banana's competitive results must could be explained by the composition variation because of different sources selected.

3.2.3. Molasses for gluconic acid production optimization

The results in Fig. 7 showed that gluconic acid production reached to 12.00 g/L at the maximum concentration of molasses. Moreover, ascribed to the samples' simultaneous utilization by molasses, resulted in great changes in cell dry weight and biomasses. The results indicate 15.50 g/L, 11.50g/L for cell dry weight, and biomasses, respectively, at 50.00 g/L of molasses. The total sugar reduction showed a drastic shift from 6 g/L to 1.40 g/l by increasing the molasses concentration.

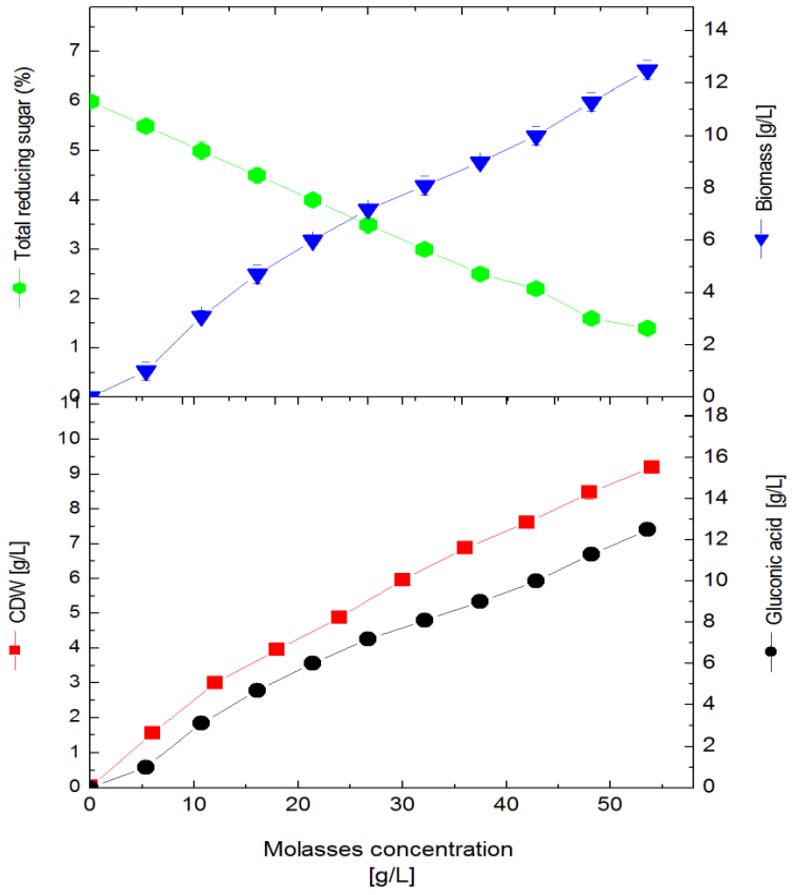

Fig. 7. Gluconic acid production after cultivation in the optimization medium contains various concentrations of molasses at 30°C for 96 h.

Although all the carbon sources considered successful in increasing gluconic acid production, it remains essential to compare them effectively. The relation between (Corn starch, Banana must, Molasses) concentrations and the culture's final pH was also an essential element in the comparison criteria. Maintaining the pH during the process was critical, and in all the carbon sources, a reduction was noticed. During the screening for the optimal raw carbon sources, a pH of 6.0 depending on gave the optimum results for *A. niger* at all the media.

Therefore, by the results obtained It is clear that molasses is better than corn starch and banana must by examining them in the optimization medium and according to the sequential results 12.00 g/L, 9.50 g/L and 6.00 g/L for production gluconic acid by *A. niger* from different feedstock as the main carbon source. Moreover, the current findings support that *A. niger* as a remarkably applicable strain for producing gluconic acid by using molasses and validate the microorganism compatibility in terms of optimization [26, 43, 44].

3.3. Kinetics of cell growth and gluconic acid production in cultivation bioreactor 4-Liter

The last phase of the study was based on the developed medium at the shake flask level. The process transferred the gluconic acid production to a larger scale of 4-L bioreactor. This step was taken as consideration toward industrialization [45].

Since the molasses was the best addition to enhance the gluconic acid, the 4-L was only investigated for molasses. Further insight into industrialization by molasses was done in a medium which is composed of g/L: Molasses 50, $NaNO_3$ 3.0,Yeast extract 3.0, $FeSO_4 \cdot 7H_2O$ 0.01, KH_2PO_4 1.0, KCl 0.5, $MgSO_4 \cdot 7H_2O$ 0.5.

The bioreactor was run under a controlled environment of PH 6.0. During the cultivation, the dissolved oxygen (DO) decreased rapidly after 3h of cultivation. The dissolved oxygen level started as 100% and was not controlled during cell cultivation process. *A. niger* growth was examined by 10% (v/v) inoculums size [16].

The optimum temperature for efficient fermentation for biomass yield and gluconic acid production from molasses by *A. niger* was set on 30ºC. This temperature is confirmed as optimal for maximal gluconic acid production by many researchers [46, 47]. The bioreactor controller measured all the parameters including temperature, pH, aeration, agitation and dissolved oxygen (DO).

Notably, gluconic production is almost 50% higher compared to shake flask culture. Besides, cell dry weight and biomass recorded a continuous escalation during the fermentation process and reached a maximal value of 27.50: 21.50 g/L, respectively, after 96 h.

Also, this value was almost 40% higher compared to the shake flask culture. The fermentation process at the 4-L bioreactor, as seen in Fig. 8, showed a gradual reduction in sugar content. The key finding is the gradual drop of sugar content from 18.40 g/L to 0.00 at 78 h.

The dissolved oxygen (DO) decreased gradually from 100% to 36.30% at the end of the process. Following the pH effect when cultivations carried out under controlled pH conditions 6.0 a considerable improvement occurred, as mentioned exactly in the literature **[**48**].**

Overall, the kinetics growth for gluconic acid production was observed. For instance, cells entered the stationary phase within 6 h. Moreover, the cells exhibited an exponential phase growth rate of 0.185 g/L.h.

Noting that the specific growth rate and the specific gluconic production reached 1.044 g/L/h and 1.475 g/g, respectively. Figure 8 summarises the key parameters attained at the 4-L bioreactor cultivation.

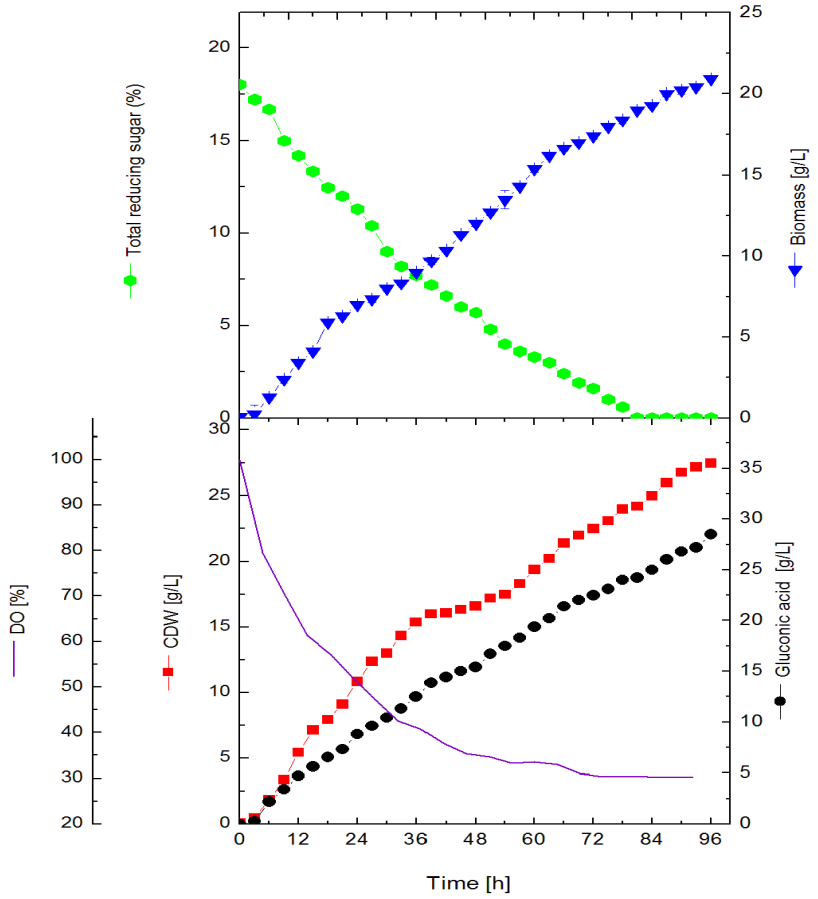

Fig. 8. Gluconic acid production, cell dry weight, pH, biomass, total reducing sugar and dissolved oxygen in 4-L bioreactor cultivation.

4. Conclusion

Gluconic acid was studied extensively in the last century for production and quality enhancement. The most important factor is finding a cost-effective approach to eliminate acid expenses. The current study found substantial evidence for *Aspergillus* fermentation optimization. Therefore, the A. *niger*, *A. fisheri*, *A. carneus* were evaluated by the mean of the total gluconic acid production. The systematic comparison showed that medium 2, which contained glucose had the highest gluconic production for all the *Aspergillus* genera studied. The *A. niger is* vouched as the best candidate. It has a gluconic acid production of 10.4 g/L. During

the optimization stage, corn starch, banana must, and molasses were contributed significantly to the addition of gluconic production.

There is a proportional relation between the concentration and the total gluconic acid production for all the carbon sources. However, the most intriguing finding is the drastic total sugar reduction among all the optimization carbon sources. The molasses showed an additional 20% enhancement of gluconic acid by *A. niger* in the absence of glucose. The molasse's outstanding performance at the end of the bioreactor stage had a maximal value of 28.50 g/L after 96 h. Notably, gluconic production is almost 50% more than the flask culture stage.

Molasses shows competitive and possible alternatives to glucose at the industrial level. Hence, the current optimization strategy can be adopted in biotechnological processes for gluconic acid production by *A. niger*. The study provided insightful information about the possibility of using commercially obtainable agro-industrial raw materials. The semi-industrial scale bioreactor 4-L results promote the method implementation for food and pharmaceutical applications.

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