



Production of Citric Acid by *Trichoderma viride* Isolated from Soil in Keffi, Nigeria Using Glucose Enhanced Substrates

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Authors' contributions

This work was carried out in collaboration between all authors. Author IKE designed the study, performed the statistical analysis, wrote the protocol. Authors MDM and MPA managed the analyses of the study and wrote the first draft of the manuscript. Authors IKE, PAT, IHN and VBO carried practical work and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This study was aimed at the production of citric acid by *Trichoderma viride* (*T. viride*) isolated from soil in keffi, Nigeria, using glucose enhanced substrate.

Place and Duration of the Study: Department of Microbiology Faculty of Natural and Applied Sciences Nassarawa State University Keffi, Nigeria, between April and June 2017.

Methodology: *Trichoderma viride* was isolated from soil in Keffi and identified using standard microbiology methods. Two types of glucose production media were prepared by following standard fermentation conditions. The citric acid produced was estimated using Gas Chromatography/Mass Spectrometry (GC/MS) method respectively.

Results: The maximum citric acid production of 12.03±0.31g/l was obtained at pH 6.0 with glucose with soybeans cake by *T. viride* and on sugar concentration of 160 g/l 15.17±3.01 g/l. The fermentation broth containing glucose and soy beans cake has the highest production of citric acid on both fermentation parameters tested respectively.

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Conclusion: Different fermentation conditions such as pH and sugar concentration substrate enhanced on the production of citric acid. This study showed that pH 6.0 with glucose with soybeans cake has highest citric acid production and at sugar concentration of 160 g/l with glucose and soybeans cake produced the highest citric acid by *T. viride*.

Keywords: *Trichoderma viride*; pH and sugar concentration; soy beans cake; groundnut cake.

1. INTRODUCTION

The organic acids are extensively used in a variety of food products as preservatives, pH adjuster, sweetness enhancer, leavening agents and stabilizers [1,2]. The acceptability of food products mainly depends on the flavour components, which are complex as well as type specific. These flavour components are influenced by the presence of organic acids and other substances like sulphur compounds, lactones, methyl ketones, alcohols and phenolic substances [3]. The important flavour substances are formed as a result of the hydrolysis of fatty acids or by the bacterial growth, or enhanced by the addition of acidulants during processing [4]. The principal organic acids having food applications are phosphoric, fumaric, tartaric, lactic and citric acids [5]. The citric acid is an important commercial product, its global production is estimated to reach 2 million tons in 2017 and its annual increasing growth rate will be 3.7% come 2018 [6,7]. The largest amount of citric acid is consumed in food industry using almost 70% of the total production, followed by about 12% in the pharmaceutical industry and 18% for other applications [8,9]. The production of citric acid by *Aspergillus niger* is one of the most commercially utilized examples of fungal overflow metabolism. Many microorganisms such as fungi and bacteria can produce citric acid.

The various fungi, which have been found to accumulate citric acid in their culture media, include strains of *Aspergillus niger*, *Aspergillus awamori*, *Penicillium restrictum*, *Trichoderma viride*, *Mucor piriformis* and *Yarrowia lipolytica* [10]. However, *Aspergillus niger* is considered as the organism of choice for the production of citric acid because of the fact that this organism has the capacity to utilize varieties of substrates due to its well-developed enzymatic system. The productivity of various fermented products is greatly influenced by the type of substrate as well as the fermentation conditions like temperature, fermentation time, pH and the type of culture/strain [11]. This study is aimed at the production of citric acid by *Trichoderma viride* isolated from soil in Keffi, Nigeria using enhanced glucose.

2. MATERIALS AND METHODS

2.1 Sample Collection

Soil samples were randomly collected (at the topsoil) from Keffi metropolis using a clean hand trowel and stored using disposable black polythene bags and transported immediately to the Microbiology Laboratory, Nasarawa State University, Keffi for analysis.

2.2 Isolation of *Trichoderma viride*

The isolation of *Trichoderma viride* was carried out following a method described by [12]. Briefly, One (1) gram of the soil sample was the suspension in a test tube containing 9 ml of sterile distilled water to make a soil suspension and ten-fold serial dilution was made by transferring one ml of the soil suspension to another test tube containing 9 ml of sterile distilled water. These steps were repeated to obtain a dilution of 10^{-7} . From each of the first three test tubes, 0.5 ml of the aliquot was spread on Potato dextrose agar plates and was incubated at 35°C for 4 days.

2.3 Identification of *Trichoderma viride*

Identification of *Trichoderma viride* was carried out as described by [12,13]. Identification was based on microbiological standard procedure using cultural and morphological characteristics. The cultural characteristics were determined by their appearance on culture plates while the morphological features were determined microscopically using lactophenol cotton blue staining technique. The isolates were identified with reference to the work of fungi standard chart.

2.4 Screening for Citric Acid-producing *Trichoderma viride*

Screening for Citric Acid-Producing *Trichoderma viride* was carried out as described by [14]. The isolates were screened qualitatively for citric acid production. Potato dextrose agar plate method containing Bromocresol green as an indicator 1%

at pH 6 was used. *Trichoderma viride* were streaked on the plates and incubate of 48 hours. Yellow zones indicate citric acid production by the test organisms.

2.5 Preparation of Inoculum for Fermentation

Preparation of inoculum for fermentation was carried out as described by [15]. Five (5 ml) of 0.3% sterile tween 80 containing in peptone water with some glass beads was transferred into four (4) days' slant culture of *Trichoderma viride* and were shaken thoroughly until spores were homogenized and incubated at 35°C for 6 hours.

2.6 Starter Culture

The starter culture was incubated as described by [16]. Seed culture 10 ml at 6 hours of inoculum was inoculated into 90 ml of freshly prepared potato dextrose broth and incubated at 35°C for 24 hours.

2.7 Fermentation Technique

2.7.1 Effect of pH on citric acid production

The batch fermentation on the effect of different ranges of pH 4.5 – 6.5 was carried out as described by [17] with modification. One hundred millimeter Medium I] containing (g/l): soybean cake 2.5 g, NH₄Cl 0.4 g, KH₂P O₄0.1 g, MgSO₄, 7H₂O 0.025 g] was added into glucose 120 g and Medium II] containing groundnut cake 2.5 g, NH₄Cl 0.4 g, KH₂PO₄ 0.1 g, MgSO₄, 7H₂O 0.025 g] were added into glucose 120, and was transferred into bioreactor used in the fermentation. The flasks were plugged with cotton and autoclaved at 15 Atm for 15 min. The sterilized flasks were inoculated with 5.0 ml of the inoculum under aseptic conditions.

2.7.2 Effect of different substrate concentration

The batch fermentation on the effect of different ranges of substrate concentration (120 – 200 g/l) was carried out as described by [18] with modification. One hundred millimeter Medium1] containing (g/l): soybean cake 2.5, NH₄Cl 0.4 g, KH₂P O₄0.1 g, MgSO₄, 7H₂O 0.025] was added into each substrate concentration and Medium 2] containing groundnut cake 2.5 g, NH₄Cl 0.4 g,

KH₂PO₄ 0.1 g, MgSO₄, 7H₂O 0.025 g] were added into each substrate concentration ranging from 120 g, 140 g, 160 g, 180 g and 200 g and was transferred into each bioreactor used in the fermentation. The flasks were plugged with cotton and autoclaved at 15 psi for 15 min. The sterilized flasks were inoculated with 5.0 ml of the inoculum under aseptic conditions. Sterilized ferrocyanide (200 ppm free ions concentration) was added to each flask incubate at 30°C for 144 hours.

The flasks were placed in a shaker incubated at a different temperature. All the experiments were run parallel in duplicates.

2.8 Analytical Methods

Sugar was estimated gravimetrically by DNS method [19]. Photoelectric colorimeter (Model: AE-11M Erma, Japan) was used for measuring colour intensity. Dry cell mass was determined by filtering the culture medium through weighed Whatmann filter paper No. 44. Mycelium was thoroughly washed with tap water and dried at 105°C for two hours.

3. RESULTS

In this study, *Trichoderma viride* isolated from soil in Keffi, Nigeria was screened for production of citric acid production using glucose with soybeans cake (gs) and glucose groundnut cake (gg) as media by submerged fermentation.

The effect of different initial pH (4.5 - 6.5) on the production of citric acid by *Trichoderma viride* was carried out as given in Table 1. The production of citric acid on different pH and glucose enhanced substrate such as glucose with soybeans (gs) and glucose with groundnut (gg) is as given below at pH 4.5, citric acid produced with gg was 6.41±0.87 g/l, were the sugar consumption was 73.02±1.20 g/l and dry cell mass was 8.68±1.18 g/l. gs produced 9.31±1.11 g/l were the sugar consumption 81.14±5.12 g/l and dry mass 8.42±1.44 g/l.

At pH 5.0 citric acid produced with gg was 7.79±1.88 g/l were the sugar consumption was 89.06±2.11 g/l and dry cell mass was 11.50±1.87 g/l. gs produced 11.12±1.34 g/l citric acid, sugar consumption of 89.06±2.11 g/l and dry cell mass 9.16±2.07 g/l.

At the initial pH of 5.5 the citric acid produced on gg was 9.01 ± 0.99 g/l, sugar consumption was 107.47 ± 5.01 g/l and dry cell mass was 12.16 ± 0.68 g/l while citric acid produced with gs was 11.87 ± 6.01 g/l, sugar consumption was 112.01 ± 5.41 g/l and dry cell mass was 9.81 ± 0.79 g/l.

Citric acid produced at pH 6.0 with gg was 10.41 ± 2.20 g/l were sugar consumption was 111.50 ± 11.10 g/l and dry cell mass was 13.61 ± 2.88 g/l while citric acid produced with gs was 12.03 ± 0.31 g/l, sugar consumption 114.20 ± 5.10 g/l and dry cell mass 10.21 ± 4.32 g/l.

At pH 6.5 the production of citric acid with gg was 9.00 ± 3.10 g/l, sugar consumption was 98.07 ± 14.02 g/l and dry cell mass 13.89 ± 1.97 g/l while citric acid produced with gs was 10.11 ± 0.88 g/l, sugar consumption was 100.24 ± 8.04 g/l and dry cell mass was 12.39 ± 3.01 g/l respectively.

Effect of different sugar concentration (120 – 200 g/l) on citric acid production by *Trichoderma viride* as given in Table 2, were the citric acid production with glucose enhanced substrate such as glucose with soy beans (gs) and glucose with groundnut (gg) is as follow at 120 g/l citric acid produced with gg was 6.91 ± 0.87 g/l, sugar consumption of 111.13 ± 0.20 g/l and dry cell mass of 13.68 ± 1.18 g/l while citric acid produced with gs was 8.81 ± 0.21 g/l, sugar consumption was 115.41 ± 1.12 g/l and dry cell mass was 9.05 ± 1.44 g/l.

At sugar concentration of 140 g/l the citric acid produced with gg was 9.19 ± 1.28 g/l sugar consumed was 131.06 ± 1.01 g/l and dry cell mass was 15.50 ± 1.87 g/l were citric acid produced in gs was 11.02 ± 2.14 g/l, sugar consumed was 136.01 ± 2.14 g/l and dry cell mass was 9.19 ± 1.28 g/l.

The citric acid produced at 160 g/l sugar concentration with gg was 13.11 ± 1.19 g/l, sugar consumed was 149.07 ± 8.01 g/l and dry cell mass was 18.16 ± 0.68 g/l while with gs the citric acid produced was 15.17 ± 3.01 g/l, sugar consumed was 155.01 ± 6.41 g/l and dry cell mass was 17.18 ± 1.49 g/l.

The maximum citric acid was produced at 180 g/l was the sugar consumption was 170.00 ± 7.10 g/l, dry cell mass was 23.61 ± 2.88 g/l and citric acid produced was 11.41 ± 1.20 g/l while with gs the

sugar consumed was 175.20 ± 5.10 g/l, dry cell mass was 19.91 ± 3.12 g/l and citric acid produced was 13.13 ± 0.81 g/l respectively.

4. DISCUSSION

The production of citric acid using glucose enhanced substrates such as glucose - groundnut cake (gg) and glucose - soybeans cake (gs) by *Trichoderma viride* and its maximum production through pH and sugar concentration optimization for citric acid production` were tested. For optimum pH, media with different pH were tested for production of citric acid and the optimum pH range for citric acid production was found to be at pH 6.0 where the amount of citric acid produced was estimated to be 12.03 ± 0.31 g/l and the citric acid produced was found to be higher in glucose enhanced medium with soybeans cake than that of groundnut cake which is in agreement with [20] reported that pH 6 was found to be optimum pH for citric acid production in similar studies. It also in agreement with earlier findings of [21,22] which states that it might be due to that at low pH, the ferrocyanide ions were more toxic for the growth of mycelium which affect the accumulation of citric acid. This finding is in agreement with [22] findings that a higher initial pH that is up to pH 8.0 leads to the accumulation of oxalic acid. In fact, a low pH in cane molasses medium has been found inhibitory for the growth of *A. niger*.

Substrate concentration is one of the most important parameters in citric acid production. The optimum citric acid produced was achieved after 144 hours of fermentation in 160 g/l of sugar concentration using *Trichoderma viride*. The maximum citric acid produced was 15.11 ± 3.01 g/l, substrate concentration 149.07 ± 8.01 g/l and dry mass was 17.18 ± 1.49 g/l. At this concentration, good amount of sugar concentration was accessible to the test organism for citric acid production starting from the initial immobile phase. The decrease in citric acid formation was observed once the sugar concentration was further increased. It might be due to mycelium overgrowth, which caused the increased thickness of the medium [23] pointed out that sugar concentration higher than 160-180 g/l leads to greater amount of residual sugars, making the process uneconomical for citric acid production, while a lower sugar concentration lead to a lower production of citric acid due to the accumulation of oxalic acid.

Table 1. Effect of pH on citric acid production by *Trichoderma viride* using glucose enhanced substrates

pH	Sugar consumed (g/l)		Dry cell mass (g/l)		Yield of citric acid (g/l)	
	Gg	Gs	Gg	Gs	Gg	Gs
4.5	73.02±1.20	81.14±5.12	8.68±1.18	8.42±1.44	6.41±0.87	9.31±1.11
5.0	89.06±2.11	92.10±3.01	11.50±1.87	9.16±2.07	7.79±1.88	11.12±1.34
5.5	107.47±5.01	112.01±5.41	12.16±0.68	9.81±0.79	9.01±0.99	11.87±6.01
6.0	111.50±11.10	114.20±5.10	13.61±2.88	10.21±4.32	10.41±2.20	12.03±0.31
6.5	98.07±14.02	100.24±8.04	13.89±1.97	12.39±3.01	9.00±3.10	10.11±0.88

Key: Substrates: gg = glucose + groundnut cake; gs = glucose + soybeans cake
*sugar concentration 120 g/l, fermentation time 144 hours and temperature of 30°C

Table 2. Effect of sugar concentration on citric acid production by *Trichoderma viride* using glucose enhanced substrates

Sugar concentration (g/l)	Sugar consumed (g/l)		Dry cell mass (g/l)		Yield of citric acid (g/l)	
	Gg	Gs	Gg	Gs	Gg	Gs
120	111.13±0.20	115.41±1.12	13.68±1.18	9.05±1.44	6.91±0.87	8.81±0.21
140	131.06±1.01	136.01±2.14	15.50±1.87	14.66±2.17	9.19±1.28	11.02±2.14
160	149.07±8.01	155.01±6.41	18.16±0.68	17.18±1.49	13.11±1.19	15.17±3.01
180	170.00±7.10	175.20±5.10	23.61±2.88	19.91±3.12	11.41±1.20	13.13±0.81
200	178.07±14.02	183.24±8.04	25.89±1.97	20.19±3.11	8.97±6.10	9.10±6.88

Key: Substrates: gg = glucose + groundnut cake; gs = glucose + soybeans cake
* Fermentation time 144 hours, temperature of 30°C and initial pH 6.0

5. CONCLUSION

Fermentation parameter for citric acid production by *T. viride* depends on the type of process. The fermentation conditions are significant to high and consistent accumulation of citric acid. In this present study, *Trichoderma viride* isolated from soil in Keffi has the capacity to produce citric acid at industrial level at pH 6.0 using glucose - soybeans cake. Also, it was observed that sugar concentration play the vital role in the production of citric acid as it observed in this study that at sugar concentration of 160g/l, *Trichoderma viride* produced the highest quantity of citric acid. The addition of soybeans cake and groundnut cake as nitrogen sources showed that it can be alternative to sources of nitrogen in the production of citric acid. *Trichoderma viride* has also shown from this study that it can compete with other fungi known to be industrial strain used in citric acid production when used for the production of citric acid under pH 6.0 and sugar concentration of 160g/l. Further study of genetic amelioration of producer strain for significant optimization of all citric acid production process from alternative substrates and subsequently citric acid production on a semi-pilot scale plant, will prove a powerful tool in the citric acid production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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