Tropical Journal of Pharmaceutical Research July 2018; 17 (7): 1235-1241
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v17i7.2

Original Research Article

Enhanced production of butyric acid by solid-state fermentation of rice polishings by a mutant strain of *Clostridium tyrobutyricum*

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Sent for review: 12 February 2018 *Revised accepted: 14 June 2018*

Abstract

Purpose: *To enhance butyric acid production by solid-state fermentation with a hyper-producing mutant of Clostridium tyrobutyricum generated by random mutagenesis.*
Methods: *Wild type C. tyrobutyricum was mutagenized with UV irradiation, nitrous acid, and ethidium bromide to obtain a hyper-producing strain. Various physiochemical parameters were optimized to increase the butyric acid yield.*
Results: *The UV-induced mutant ($C.T^{UV}$) produced significantly higher concentrations of butyric acid than the wild type parent, nitrous acid-induced, and ethidium bromide-induced strains. $C.T^{UV}$ increased butyric acid production 1.4-fold more than the parent strain. Fermentation with $C.T^{UV}$ with 2.5 g of rice polishings (w/w), a 2 % inoculum volume (v/v), and a 48-h incubation period at 37°C under anaerobic conditions produced 11.63 mg/100 g of butyric acid. The addition of 0.6 % corn steep liquor as a nitrogen source increased the butyric acid concentration to 26.09 mg/100 g.*
Conclusion: *These optimized fermentation parameters on a small scale can be used on a commercial scale to mass-produce butyric acid.*

Keywords: *Butyric acid, Mutant, Clostridium tyrobutyricum, Mutagen, Solid-state fermentation*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Butyric acid is an ill-smelling, 4-carbon, short chain fatty acid, $CH_3CH_2CH_2COOH$, and is an important solvent, polymer, and chemical compound used in the pharmaceutical, food, and chemical industries and is extensively used in thermoplastics. As a raw material, it is used to produce the biodegradable polymer β-hydroxybutyrate [1]. It is also used in the treatment of colon cancer and as an energy source for the human body [2], and butyric acid esters give tropical fruits and dairy products their flavors [3, 4]. Currently, there is great interest in using butyric acid as a precursor to biofuels.

On a commercial scale, butyric acid is produced through an oxy-fuel combustion process with

petrochemicals as substrates [5]. The manufacturing of renewable chemicals by microbes has been stimulated by increases in oil prices, petrochemical industry environmental pollution, and consumer interest in the inclusion of natural ingredients in cosmetics, foods, and pharmaceuticals [6-10]. Although numerous Gram-positive obligate anaerobes, such as *Clostridium spp.* and *Butyrivibrio fibrisolnes,* produce significant amounts of butyric acid during fermentation [11], *Clostridium* strains are favored in industry because of their higher outputs and final-product concentrations [12]. *Clostridium tyrobutyricum* is one of the most preferred strains due to its high and stable production of butyric acid [13-15].

The cost to produce butyric acid by fermentation depends on the cost of the substrate, and it is costly to use pure carbon sources as substrates. The use of economically feasible and renewable substrates, such as agricultural products, as substrates has been investigated [16]. Agro-industrial wastes that are produced in bulk during milling include rice polishings, wheat bran, and molasses.

This is the first study that utilized rice polishings for butyric acid production by solid-state fermentation (SSF). More common processes for butyrate production are batch, fed-batch, continuous, and cell-recycle fermentations, but SSF is more economical due to low water requirements, reduced downstream processing, and low stirring requirements [17].

Due to the interest in using butyric acid as a biofuel precursor, this study aimed to hyper-produce butyric acid by creating a stable *C. tyrobutyricum* mutant and by optimizing fermentation parameters.

METHODS

Bacterial strain and medium

The parent strain *C. tyrobutyricum* DSM 2637 (ATCC 25755 NCIB 110635) was procured from DSMZ, Germany. The organism was revived and maintained under anaerobic conditions on Reinforced Clostridial Medium (RCM) agar plates and slants.

Random mutagenesis

C. tyrobutyricum DSM 2637 was exposed to various physical and chemical mutagens to create a mutant with enhanced butyric acid production.

UV irradiation

One mL of a fresh *C. tyrobutyricum* culture was transferred to RCM agar plates, and the plates were exposed to UV irradiation (Model: Mineral light UVS-12, California, USA) for various amounts of time (10–60 min). By adjusting the distance between the UV lamp and the culture plate to 5.0 cm, a death rate of 95% was achieved [18].

To avoid photostimulation, the UV irradiated cells were grown in the dark in an anaerobic chamber at 37°C for 48 h, colonies were counted, survival curves were generated, and colonies were screened for butyric acid production [15].

Nitrous acid (NA) treatment

NA solutions of various concentrations were prepared by adding 0.9 mL of 0.1 M acetate buffer (pH 6.8) to 0.1 mL of varying concentrations of sodium nitrite (1, 2, 3, 4, or 5 M). Cell pellets of freshly grown 18 h cultures of *C. tyrobutyricum* were washed with 0.2 M phosphate buffer pH 7.0 and incubated with NA for various amounts of time (20, 40, or 60 min) at 37°C [19]. The cells were then centrifuged and the cell pellets were washed with phosphate buffer to remove traces of the mutagen.

After washing, the cell pellets were resuspended in 1 mL of 0.2 M phosphate buffer pH 7.0, and serial dilutions were prepared and spread onto RCM agar plates. The plates were incubated in an anaerobic chamber at 37°C for 48 h, colonies were counted, and survival curves were generated. Plates with a death rate >90% were selected and colonies were screened for butyric acid production [15].

Ethidium bromide (EtBr) treatment

Cell pellets of freshly grown 18 h cultures of *C. tyrobutyricum* were resuspended in 2 mL of saline solution and incubated with varying concentrations of ethidium bromide (15, 18, 21, 24, 27, or 30 mg/mL) at 37°C for 1 h. The cells were centrifuged at 10,000 rpm for 5 min, resuspended in 1 mL of 0.2 M phosphate buffer pH 7.0, and serial dilutions were prepared and spread onto RCM agar plates.

The plates were incubated in an anaerobic chamber at 37°C for 48 h, colonies were counted, and survival curves were generated. Plates with a death rate >90% were selected and colonies were screened for butyric acid production [15].

Fermentation of wild type and mutant *C. tyrobutyricum*

Single wild type and mutant *C. tyrobutyricum* colonies were transferred from fresh RCM agar plates to 250 mL Erlenmeyer flasks containing 50 mL of RCM broth and incubated at 37°C for 24 h with shaking at 200 rpm. The cultures were diluted to an OD_{600} of 0.6 with RCM medium, added to fermentation media, and incubated under anaerobic conditions at 37°C for 72 h. The fermentation medium contained 0.25% (v/w) NaCl, 0.25% $CaCl_2$, 0.3% $MgSO_4$, 0.15% KH_2PO_4, 0.5% corn steep liquor, and 2.5 g rice polishings at pH 6.0.

Optimization of parameters for butyric acid production

To increase the butyric acid yield, various fermentation parameters were tested. The parameters tested included the substrate:water ratio (10:24–10:44), the volume of the inoculum (1–3 mL), incubation time (2–8 days), the NaCl concentration (0.125–30%), the $CaCl_2$ concentration (0.125–0.30%), the $MgSO_4$ concentration (0.175–0.35%), the KH_2PO_4 concentration (0.05–0.25%), the nitrogen:carbon ratio (1:10–1:30), and the corn steep liquor concentration (0.2–0.8%). These fermentations were performed in 250 mL Erlenmeyer flasks containing 11.5 mL of medium under anaerobic conditions at 37°C. The variations were tested in triplicate and were expressed as averages.

Quantification of butyric acid

Quantification of butyric acid was carried out by the organic analysis method [20], which is based on the catalytic oxidation of butyric acid into diacetic acid, which gives a red color upon the addition of sodium nitroprusside. Five mL of a fermented liquor sample was shaken first with 5 mL of a hydrogen peroxide solution, then shaken with one mL of a 5 % ferrous ammonium sulphate solution, followed by shaking with 10 mL of a 10 % sulfuric acid solution. The mixture was heated for 5 min in a water bath at 68 – 70 °C. Then, six drops of 20 % sodium hydroxide were added to the cooled mixture and the mixture was filtered. Three drops of a 20% sodium hydroxide solution, three drops of a 5 % sodium nitroprusside solution, and 0.5 mL of acetic acid were added to 5 mL of filtrate. In the presence of butyric acid, a rosy red color is produced, which can be measured spectrophotometrically by the absorbance at 390 nm. The Pearson correlation coefficient was used to evaluate the relationship between OD_{390} and butyric acid concentration and showed that there is a positive linear correlation between OD_{390} and butyric acid concentration.

Statistical analysis

Data were analyzed statistically using SPSS 16.0 software and were presented as mean ± SD of three replicates Means were compared by One-Way ANOVA, descriptive analysis, and least significant difference (LSD). $P < 0.05$ were considered significant.

RESULTS

Effect of physical and chemical mutagens on wild type *C. tyrobutyricum*

After exposing wild type *C. tyrobutyricum* to UV irradiation, NA, and ethidium bromide, colonies from plates with a >90 % death rate were selected. The survival curves for each mutagenesis treatment are shown (Figure 1A). Mutant strains were screened for increased

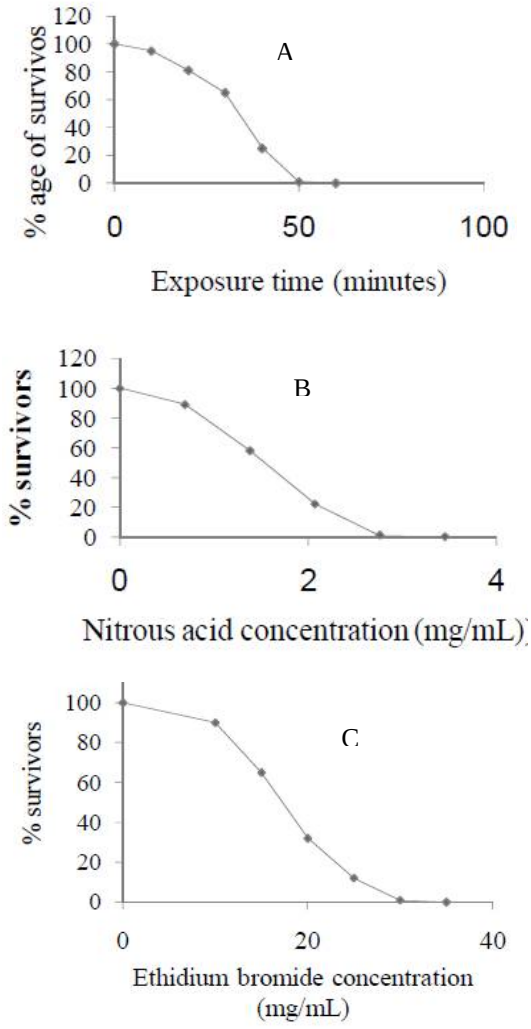

Figure 1: *C. tyrobutyricum* survival curves after treatment with physical and chemical mutagens: (A) UV irradiation, (B) Nitrous acid, (C) Ethidium bromide

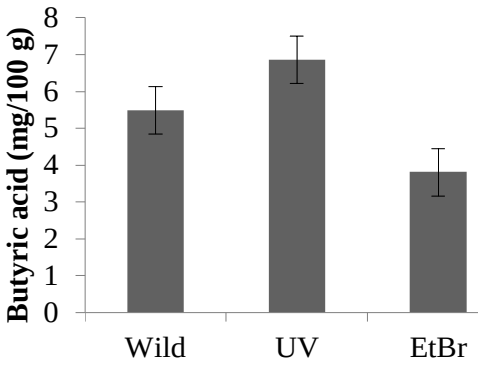

Figure 2: Mean concentrations of butyric acid produced by mutant *C. tyrobutyricum* strains. UV = Ultraviolet radiation-induced mutant; EtBr = Ethidium bromide-induced mutant; N.A = Nitrous acid-induced mutant

Table 1: Statistical analysis of mean concentrations of butyric acid produced by mutant *C. tyrobutyricum* strains

Yield (mg/100 g)	Mutant			
	Wild (control)	**UV**	**EtBr**	**NA**
Butyric acid	5.48±0.274^{b}	6.79±0.178^{a}	3.85±0.040^{d}	4.74±0.098^{c}

**Means with different superscripts differ significantly ($p < 0.05$)*

butyric acid production using previously optimized fermentation conditions. The mutant strain generated with UV irradiation (*C.T^{UV}*) produced 6.86 mg/100 g butyric acid, the mutant strain generated with ethidium bromide produced 3.81 mg/100 g butyric acid, and the mutant strain generated with NA produced 4.73 mg/100 g butyric acid, whereas the wild type strain produced 5.49 mg/100 g butyric acid (Figure 2, Table 1)**.**

Effect of various physiochemical parameters on butyric acid production

We previously determined that rice polishings (2.5 g) are an optimal carbon source for wild type *C. tyrobutyricum* fermentation, thus we used rice polishings as the carbon source while optimizing other parameters, including the substrate:water ratio, the incubation period, and the inoculum volume, during SSF of the *C.T^{UV}* mutant strain at 37°C and pH 6.0. Of the various substrate:water ratios, the 10:36 substrate:water ratio gave the highest butyric acid yield of 7.47 mg/100 g (Figure 3B), and of the various incubation periods, 3 days of incubation gave the highest butyric acid yield of 6.86 mg/100 g (Figure 3A). Of the various inoculum volumes tested, 2.5 ml of inoculum gave the highest butyric acid yield of 7.69 mg/100 g, whereas an increase in the inoculum to 3 mL reduced the butyric acid yield to 4.61 mg/100 g (Figure 3C).

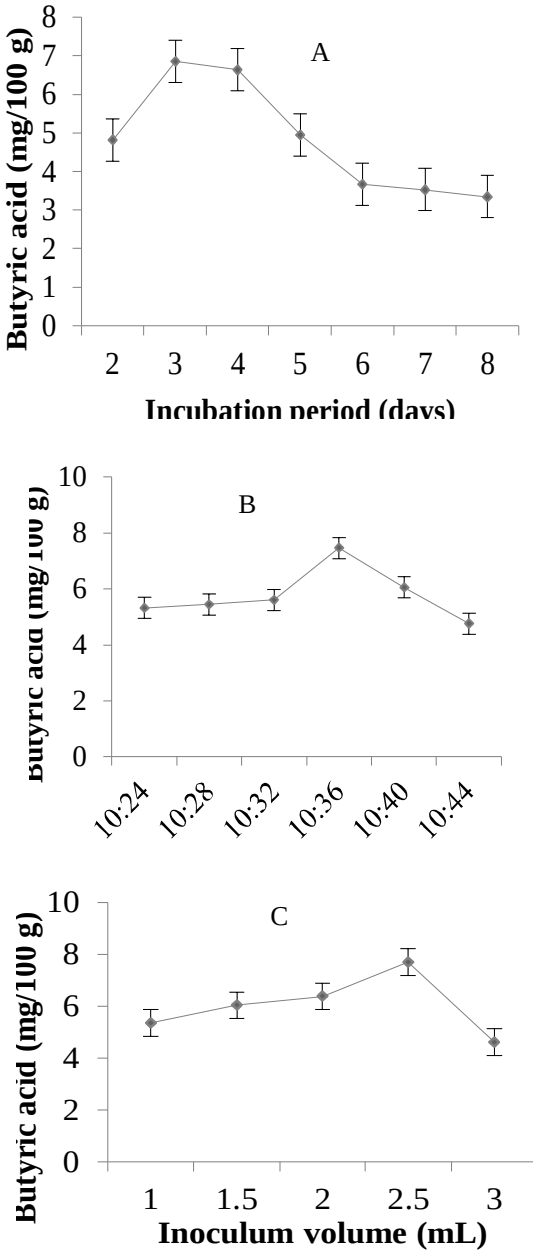

Figure 3: The effects of various parameters on butyric acid production during SSF of rice polishings by the mutant *C.T^{UV}* at pH 6.0 and 37°C: (A) Incubation period, (B) Substrate:water ratio, (C) Inoculum volume.

Various concentrations of NaCl, $CaCl_2$, $MgSO_4$, and KH_2PO_4 were tested to determine optimal salt concentrations for the highest butyric acid yield during SSF of rice polishings by the mutant *C.T^{UV}* at pH 6.0 and 37°C. The salt concentrations of 0.25% (v/w) NaCl, 0.25% $CaCl_2$, 0.3% $MgSO_4$, and 0.15% KH_2PO_4 yielded the highest butyric acid concentrations of 8.8 mg/100 g, 9.6 mg/100 g, 10.52 mg/100 g, and 10.66 mg/100 g, respectively (Figure 4 A-D).

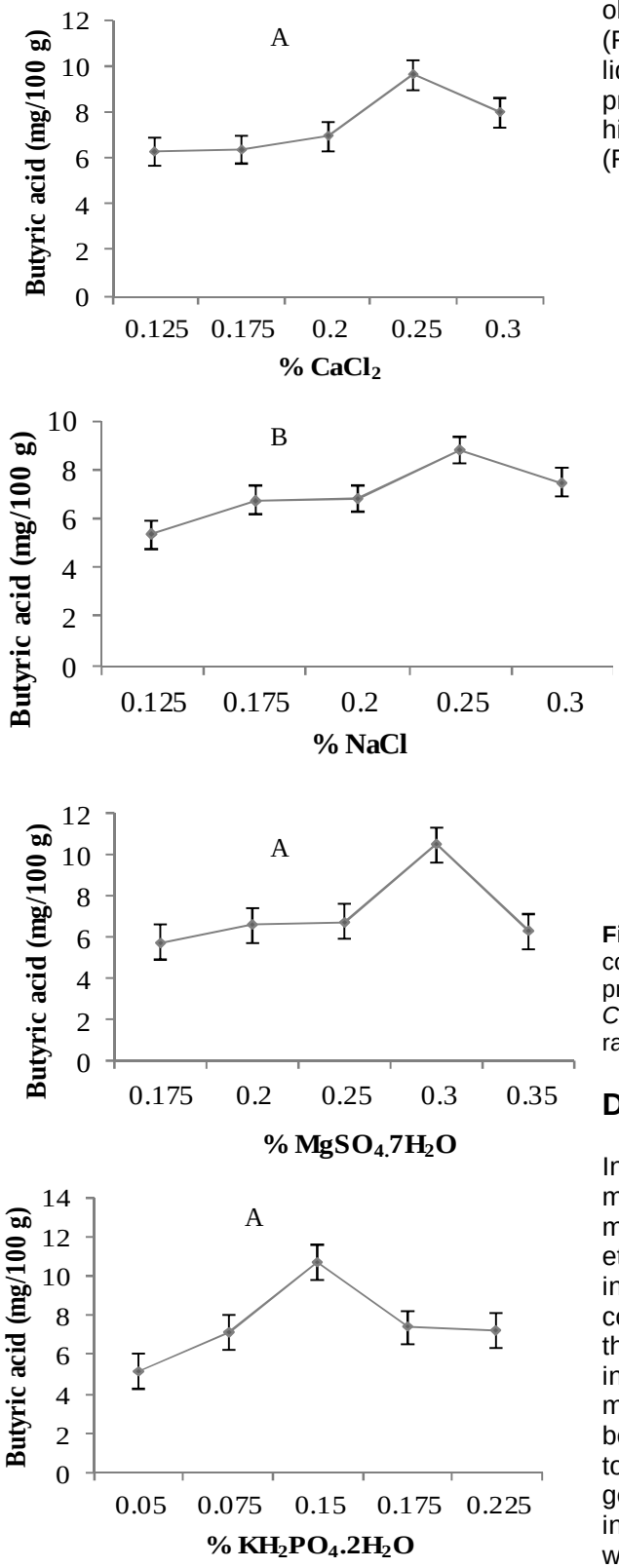

Figure 4: Effects of various salt concentrations on butyric acid production during SSF of rice polishings by mutant *C.T*^{UV} at pH 6.0 and 37°C: (A) NaCl, (B) $CaCl_2$, (C) $MgSO_4$, (D) KH_2PO_4.

Various nitrogen:carbon ratios were investigated for maximum butyric acid production. The highest butyric acid yield of 11.63 mg/100 g was observed with a nitrogen:carbon ratio of 1:25 (Figure 5A). Various concentrations of corn steep liquor were also tested for maximum butyric acid production, and 0.6% corn steep liquor gave the highest butyric acid yield of 26.09 mg/100 g (Figure 5B).

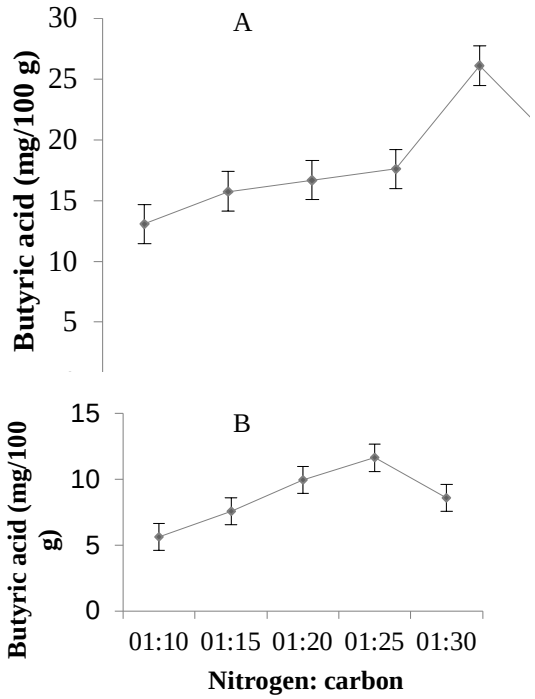

Figure 5: Effects of the nitrogen:carbon ratio and the corn steep liquor concentration on butyric acid production during SSF of rice polishings by mutant *C.T*^{UV} at pH 6.0 and 37°C: (A) the nitrogen:carbon ratio, (**B)** corn steep liquor

DISCUSSION

In this study, we created three *C. tyrobutyricum* mutants by random mutagenesis, a UV-induced mutant, a nitrous acid-induced mutant, and an ethidium bromide-induced mutant. The UV-induced mutant produced significantly higher concentrations of butyric acid when compared to the ethidium bromide-induced and nitrous acid-induced mutants, and therefore the UV-induced mutant *C.T*^{UV} was used in further studies. It has been reported that exposure of *C. tyrobutyricum* to heavy-ion irradiation with $^{12}C^{6+}$ led to the generation of a *C. tyrobutyricum* mutant that increased butyric acid production from 2.2 g in wild type to 3.3 g in the mutant [21]. The mutant *C.T*^{UV} significantly increased the butyric acid yield after 3 days of incubation and with a 10:36 substrate:water ratio. The volume of the inoculum generally affects the amount of product produced by fermentation. Previous studies found that 50 mL and 100 mL *C. tyrobutyricum*

inoculum volumes increased butyric acid production by 5% (v/v) to 34.2 g/L and 3.87% to 4.3 g, respectively [10,12,22]. In this investigation, a significantly higher concentration of butyric acid was produced with a 2.5 % (v/w) *C.T*^{UV} inoculum, whereas the maximum butyric acid yield with wild type *C. tyrobutyricum* was observed with a 2 % (v/w) inoculum.

Butyric acid production may also be affected by the nutritional value of the medium. Rich media usually enhance butyric acid production. More butyric acid was produced by *C. tyrobutyricum* fermented in RCM than in Clostridial Growth Medium [8,23]. A medium containing 500 g/L glucose, 25 g/L $MgSO_4$, 1.3 g/L $MnSO_4$, and 0.6 g/L $FeSO_4$ has been recommended to increase butyric acid production [21]. It has also been shown that a nutrient formulation composed of 30 g/L glucose, 5 g/L yeast extract, 5 g/L peptone, 3 g/L $(NH_4)_2SO_4$, 0.6 g/L $MgSO_4$, and 0.03 g/L $FeSO_4$ increased butyric acid production [24]. Further, the addition of corn steep liquor with corn fiber hydrolysate as a substrate has been shown to increase the butyric acid yield [25]. In this study, we found a *C. tyrobutyricum* mutant strain that significantly increased butyric acid production 1.4-fold when compared to the wild type *C. tyrobutyricum* strain.

CONCLUSION

An SSF technique for butyric acid production using rice polishings as the substrate has been developed. The *C.T*^{UV} mutant increased butyric acid production to 26.09 mg/100 g, which is 1.4-fold more than the butyric acid produced by the wild type parent strain, thus this simple and inexpensive method can be used for butyric acid production on a commercial scale.

DECLARATIONS

Acknowledgement

The authors would like to thank Dr. Masood Rabbani, Dr. Fareeha, Ms. Huma Mujahid, and the University Diagnostic Laboratory (UDL) for providing the facilities to perform the random mutagenesis procedures.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Ms. Tasleem Akhtar, Dr. Abu Saeed Hashmi, Dr. Muhammad Tayyab, and Aftab Ahmed Anjum conceived and designed the study. Ms. Tasleem Akhtar collected and analyzed the data and wrote the manuscript. Dr. Shagufta Saeed assisted in the writing of the manuscript. All of the authors have read and approved the manuscript for publication.

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