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RESEARCH PAPER

Bench scale production of butyrohydroxamic acid using amidotransferase activity of amidase from whole resting cell *Bacillus* sp. APB-6.

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Abstract

Butyrohydroxamic acid is a hydroxamic acid that has various biological and pharmacological applications. This study reports the bioconversion of butyramide and hydroxylamine to butyrohydroxamic acid with the help of amidase of Bacillus sp. APB-6, which has amidotransferase activity. Optimal conditions for the reaction were determined as 100/1200 mM butyramide/hydroxylamine ratio, incubation time 5 hr, pH 9.5, temperature 55°C, and resting cell concentration of 1.578 mg dcw ml-1. Under these conditions, the complete conversion of butyramide to butyrohydroxamic acid was attained in a 50 ml flask scale. The batch reaction was preferred over fed-batch reaction for scaling up the process to a 1 L scale, and the reaction time was reduced by 30 minutes. The final product yield was 10.23 g butyrohydroxamic acid with 95% purity, volumetric productivity of 2.273 g/L/h and 1.44 g/g/h catalytic productivity. The amidase used in this study showed high amidotransferase activity along with the industrially relevant process for the production of butyrohydroxamic acid. The NMR spectrum of the recovered product confirmed its identity as butyrohydroxamic acid.

Introduction

In recent years, there has been tremendous interest in the application of hydroxamic acids and their derivation. They have pharmacological and toxicological significance and have been shown to have various biological activities such as antimicrobial, antileukemic, antituberculous, and chemotherapeutic agents (Syed et al., 2020). Also, α-amino hydroxamic acid derivatives can effectively block the activity of metalloproteases, which are responsible for the tissue changes that facilitate tumor development and metastasis (Lin et al., 2022). Also, acetohydroxamic acid and butyrohydroxamic acid have shown anti-human immunodeficiency and antimalarial activities (Adebayo et al., 2024; Wang et al., 2022; Končić et al., 2011). The chemical synthesis of hydroxamic acids usually involves the Angeli-Rimini reaction or Lossen rearrangement (Mountanea et al., 2023). However, these methods need many solvents and complex reaction conditions, such as nitrogen requirements, higher temperatures, and tedious steps that may produce undesired by-products (Wang et al., 2022). On the other hand, mild reaction conditions can be achieved with the use of

enzymes, and therefore find more applications as biochemicals.

The main determining factors for the effective application of enzyme-based bioprocesses to useful products are enzyme activity, substrate, and product tolerance (Victorino da Silva Amatto et al., 2022; Kanwar et al., 2024). Amidotransferases are of considerable industrial interest in bioconversion processes to make valuable hydroxamic acids. Researchers have exploited amidases with acyl transfer activities for the enzymatic conversion of amides to hydroxamic acid. (Wu et al., 2020; Sharma et al., 2022). Hydroxamic is synthesized by enzymes under mild conditions of pH, temperature, and pressure, resulting in a pure product. These bioprocesses are more advantageous than the current chemical processes, as they offer high specificity, high selectivity, and environmental friendliness (Boodhoo et al., 2022). To overcome the challenges of large-scale hydroxamic acid production, this study focuses on optimizing fermentation parameters for butyrohydroxamic production. Here, we described the amidotransferase activity of the amidase of *Bacillus* sp. APB-6 with high butyramide tolerance. We optimized the process at a 50 mL scale using fed-batch and batch reactions, and at a bench scale (1L), which can be further upscaled to industrial-level bioreactors. To the best of the literature knowledge, this is the first study exploiting bacterial amide for the batch-scale production of butyrohydroxamic acid from *Bacillus* sp. APB-6.

Materials and Methods

Reagents and chemicals

The amides were procured from Alfa Aesar (Heysham, UK) (A Johnson Matthey Company). All other chemicals, including the culture media and their components, were purchased from HiMedia (Mumbai, India).

Bacterial strain and growth parameters

The bacterial strain APB-6 was obtained from the Department of Biotechnology, Himachal Pradesh University, isolated in a previous study, and acquired accession number MTCC-7540 (Pandey et al. 2011). The strain was cultured in a modified nutrient broth as per described by Kumari and Chand (2017) and Kumari et al. (2017).

Amidotransferase assay

Amidotransferase activity was measured as per the previous method of Brammar and Clarke (1964). To prepare the standard curve, butyrohydroxamic acid was used in concentrations ranging from 80 mg to 340 mg. Amidotransferase activity was measured in terms of enzyme units (U), where one unit corresponded to the quantity of enzyme that released one micromole of product per min under standard reaction conditions (Kumari et al., 2017).

Analytical methods

Substrate and product were subjected to high-performance liquid chromatography (HPLC) for their quantitative estimation using the Waters HPLC system, which consisted of a reverse phase C18 column with 4.6 mm x 250 mm; 5 µm dimensions and a 515 series pump operated at 1 mL/min flow rate. The solvent system consists of 30% acetonitrile-water with 0.2% orthophosphoric acid. Absorbance was recorded at 200 nm using a photodiode array (PDA) detector. The standard curves were prepared for butyramide (20-200 mM), hydroxylamine (50-200 mM), and butyrohydroxamic acid (1-10 mM).

Effect of substrate molarity and enzyme concentration

The optimization of the conversion of butyramide to butyrohydroxamic acid was performed by varying the concentrations of butyramide (100 mM, 150 mM, and 200 mM) and resting cell (0.789-2.367 mg dcw/mL) as a catalyst and keeping the hydroxyl amine concentration constant (1200 mM). The reaction was conducted at 50 mL scale for 120 min at 55°C.

Time course for enzymatic reaction at variable temperatures

The temperature dependency of enzyme activity was studied by stopping the reaction at 2, 4, 6, and 8 hrs intervals at 50°C, 55°C, and 60°C variable temperatures. The enzymatic reaction was performed at a 50 mL scale with 1200 mM hydroxylamine. The product concentration (mM) was determined in HPLC by comparing it against the commercial standard of butyrohydroxamic acid.

Time course of butyramide conversion for increasing substrate concentration

To study the effect of butyramide concentration, a reaction was set using 50 mM-200 mM butyramide and 1200 mM hydroxyl amine. The reaction was conducted on a 50 mL scale at 55°C. The samples were analyzed hourly by HPLC for substrate and product concentrations.

Time course of butyramide conversion with a corresponding increase in the concentration of substrate and resting cell

Optimization of the reaction volume (50 mL) was performed by increasing the concentrations of substrate and resting cell proportionally from 100 mM; 2.104 mg dcw/mL to 150 mM; 3.156 mg dcw/mL and 200 mM; 4.206 mg dcw/mL. The reaction was analyzed by HPLC after 5 hrs for substrate and product concentrations.

Standardization of 50 mL scale fed-batch and batch reactions

A 50 mL scale batch reaction was performed with 0.2 M of glycine-NaOH having a pH of 9.5, 100 mM butyramide, 1200 mM hydroxylamine, and 1.578 mg dcw/mL resting cells at 55°C. The reaction was shaken

reciprocally in a water bath for 5 hrs and analyzed by HPLC for substrate and product concentrations.

To increase the molar conversion yield, a 50 mL scale fed-batch reaction was performed using 0.2 M Glycine-NaOH buffer with pH 9.5, 100 mM butyramide, and 1200 mM hydroxylamine along with resting cells (1.578 mg dcw/mL) at 55°C. Further, the flask containing the reaction was shaken reciprocally in the water bath and the substrates (butyramide 100 mM and hydroxylamine 1200 mM) were added every 5 hrs. Three additions were made and the product concentration was measured by HPLC before and after each addition.

Bench scale (1L) bioconversion of butyramide and hydroxylamine to butyrohydroxamic acid

Bench scale (1L) bioconversion was performed in a BIOFLO C-32 fermenter (New Brunswick Scientific, Edison, NJ, USA) with a 1.5 L capacity. The reaction conditions comprise 0.2 M glycine NaOH having 9.5 pH, 100 mM butyramide, 1200 mM hydroxylamine, and resting cells with 1.578 mg dcw/mL concentration at 55°C. Further, the substrates and resting cells were mixed well at 200 rpm. The reaction was conducted for 5 hrs, and at one-hour interval, samples were analyzed by HPLC for the concentration of substrate and product.

Downstream processing of the reaction mixture

The reaction mixture was then centrifuged at 10,000 g for 15 min at room temperature to remove the resting cells from the supernatant. After this, the supernatant was vacuum-dried in a lyophilizer to reduce its volume by half and then used for acid-base extraction. Further, the supernatant was acidified with 12.5 N HCl and resuspended in 20 mL of acetone. To eliminate the salts the solution was filtered using Whatman filter paper and the filtrate was evaporated to obtain a slightly white powder. The weight of the powder was measured to calculate the recovery percentage. The powder was analyzed by HPLC to check its purity (Fournand et al., 1997). For characterization, the purified product was dissolved in deuterated dimethyl sulfoxide and an NMR spectrum was obtained to confirm butyrohydroxamic acid (Figure 1).

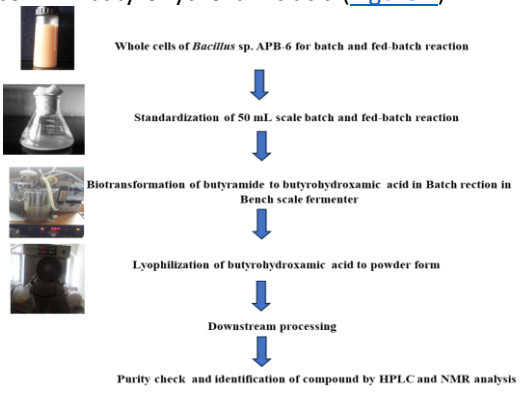


Figure 1. Schematic for bench scale biotransformation process of butyramide to butyrohydroxamic acid.

Results and Discussion

Effect of substrate molarity and enzyme concentration using resting cells

The conversion of butyramide to butyrohydroxamic acid was performed by varying the resting cell concentration and the butyramide concentration while keeping the hydroxylamine concentration constant (1200 mM). The conversion rate increased with the increase in cell concentration, and the highest conversion was achieved with 1.578 mg dcw for resting cells and 100 mM butyramide in 2 hrs (Figure 2).

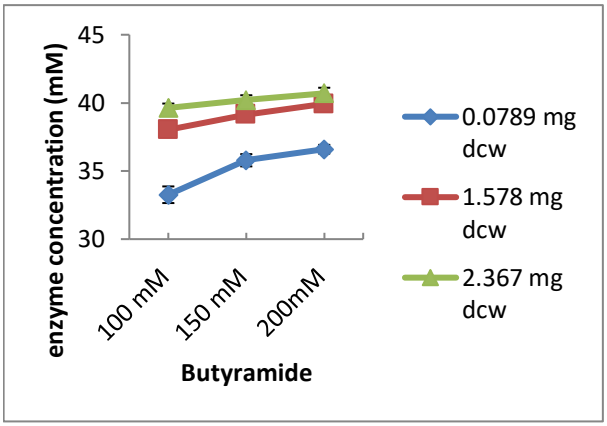


Figure 2. Effect of molarity of substrate and enzyme concentration on conversion of butyramide to butyrohydroxamic acid.

Therefore, these conditions were selected for the subsequent experiment. However, at 2.367 mg dcw/mL, the conversion of 100 mM substrate was only slightly higher (39.63%) than that at 1.578 mg dcw/mL (38.03%) in 2 hrs using resting cells. This suggests that the further addition of cells did not contribute to a significant increase in conversion. For the prevention of hydrolysis of the product and process cost-effectiveness, 1.578 mg dcw/mL was chosen for the subsequent experiment. These results were supported by Agarwal et al. (2013), who found an increase in conversion rate with the increase in enzyme concentration during the bioconversion of nicotinamide to nicotinic acid using the acyltransferase activity of a bacterial strain, and the maximum conversion was observed at 0.7 mg dcw/mL resting. Similarly, Pandey et al. (2011) used 300 mM of acetamide and 800 mM of hydroxylamine to produce the acetohydroxamic acid using resting cells of *Bacillus* sp. Likewise, a study by Devi et al. (2022), reported optimized conditions for the bioconversion process of acetohydroxamic acid using the acyltransferase activity of *Rhodococcus pyridinivorans* using 300 mM of acetamide and 500 mM of hydroxylamine-HCl which led to product conversion a rate of 89% at 45° C in a 30 min reaction (Devi et al., 2022).

Effect of the time course of enzyme reaction using resting cells at different temperatures

The conversion of butyramide to butyrohydroxamic acid was faster at higher temperatures and time. At 50°C, the conversion was slow, but at 55°C, 100 mM butyramide was completely converted within 5 h for resting cells (Figure 3). Devi et al. (2022), reported 60°C as the optimum temperature for the activity of acyltransferase using resting cells The highest acyltransferase activity of strain APB-6 was observed at 45°C.

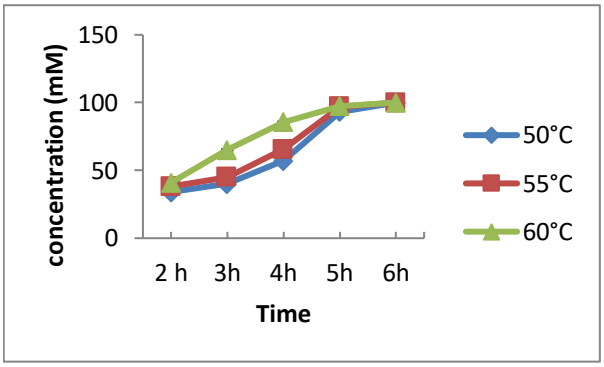


Figure 3. Time course of butyrohydroxamic acid production at different temperatures using resting cells.

Similar results were obtained during the bioconversion process of nicotinamide to nicotinic acid by using the acyltransferase of strain IITR6b2, where the highest conversion was achieved at 55°C (Agarwal et al., 2013).

Effect of the time course of butyramide conversion with a corresponding increase in substrate concentration

We have observed an increase in product formation about increasing substrate concentration along with time. Butyramide with 50 mM and 100 mM concentrations was completely converted within 5 h with resting cells (Figure 4).

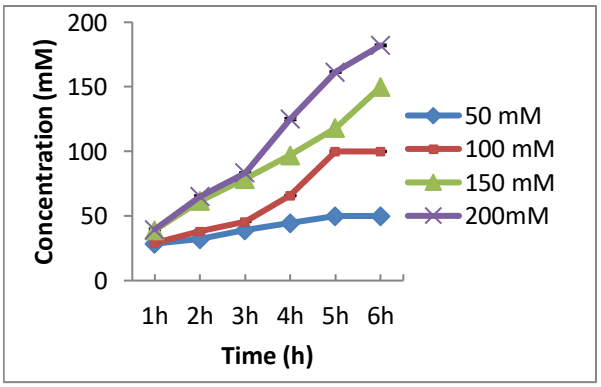


Figure 4. Time course of butyrohydroxamic acid production at various substrate concentrations using resting cells.

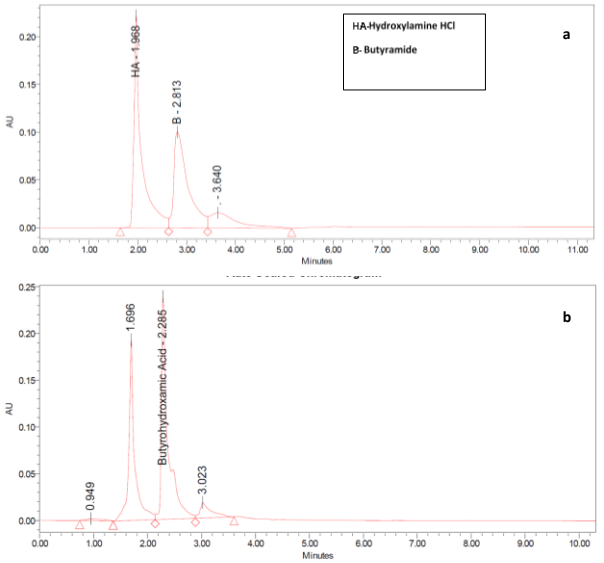
Singh et al. (2020), reported the time course for bioconversion of N'-phenyloctanediamide (10 mmol l^{-1}) to N-hydroxy-N'- phenyloctanediamide and reported maximum conversion after 12 hr at 40°C. Whereas, maximum conversion of acetamide (300 mM), and hydroxylamine-HCl (800 mM) was observed after 30 min at 45°C by using the acyltransferase activity of *Rhodococcus pyridinivorans* (Devi et al., 2022).

Time course of butyramide conversion with a corresponding increase in substrate and resting cell concentration

The product formation increased with an increase in the concentration of butyramide and resting cells. The highest conversion was achieved with 1.578 mg dcw/mL and 100 mM butyramide. Devi et al. (2022), reported optimum conversion using resting cells with 576 U/mg-dcw residual activity using 300 mM of acetamide.

Batch and fed-batch reactions to produce butyrohydroxamic acid using resting cells

Batch and fed-batch reactions on a 50 mL scale were done in a 250 mL Erlenmeyer flask to increase the molar yield. HPLC analysis of commercial hydroxylamine HCl, butyrohydroxamic acid, and butyramide was done. The zero-hour sample (before the reaction started) showed the peaks of both substrates (butyramide and hydroxylamine HCl) in HPLC (Figure 5a).



Sr. No.	Name	RT	Area	Height
1.	Hydroxylamine HCl	1.696	1437875	193232
2.	Butyrohydroxamic Acid	2.285	2013205	238412

Figure 5. HPLC chromatogram of zero h sample (a), and HPLC chromatogram of butyrohydroxamic acid in batch reaction (b).

The batch reaction using resting cells produced 100% butyrohydroxamic acid and the total product concentration was 100 mM at 50 mL scale. The HPLC chromatogram showed the peaks of butyramide (product) and hydroxylamine, which were used in excess (1:12) compared to butyramide and remained unreacted (Figure 5b).

The product concentration before and after each feeding in the fed-batch reaction was measured by HPLC. The fed-batch reaction was not successful, as the conversion rate decreased after the feeding, even though the total product amount increased. Before the

feeding of substrates (after 5 hrs), 100 mM butyramide was completely converted to butyrohydroxamic acid (Figure 6). Bhatia et al. (2013), performed a 50 mL scale fed-batch reaction and applied five feedings of benzamide (50 mM) and hydroxylamine (250 mM) at time intervals of 20 min for benzohydroxamic acid production at 50°C.

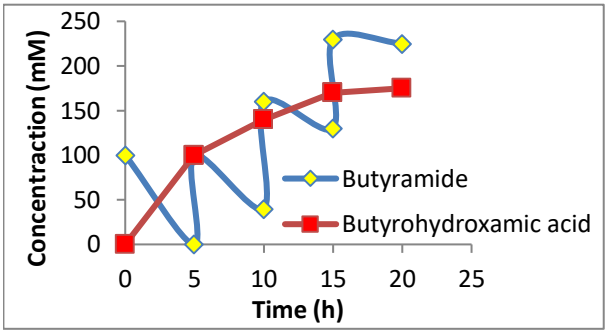


Figure 6. Synthesis of butyrohydroxamic acid using resting cell in 50 mL scale fed-batch reaction.

Also, a thermostable amidase from *Xinfangfangia* sp. DLY26 with optimal activity at 60°C and broad substrate specificity was reported by Xi et al. (2021). The maximum product formation was observed at the fourth feeding. After the feeding of substrates (after 10 hrs), the conversion was only 40%. Whereas, the result obtained in batch reaction with a tremendous conversion rate highlights the importance of the current study. This is the highest conversion rate report for butyrohydroxamic acid production. Therefore, the results further motivate us to upscale to bench scale (1L) for the synthesis of butyrohydroxamic acid.

Bench scale (1L) bioconversion of butytamide and hydroxylamine to butyrohydroxamic acid

A bench scale (1 L) process was performed in a fermenter vessel with a temperature of 55°C and an impeller speed of 200 rpm (Figure 7).

Figure 7. New Brunswick Scientific BioFlow C-32 fermenter (1.5 L) showing the reaction containing resting cells of APB-6 for the biotransformation of butyramide to butyrohydroxamic acid.

The reaction lasted for 5 hrs and produced 100 mM butyrohydroxamic acid (100% molar conversion yield). The conversion of 100 mM butyramide to butyrohydroxamic acid was almost complete within four and a half hours, which was faster as compared to 50 mL reaction. The reason behind this may be the kinetic change in the reaction and increased interaction surface area of the enzyme and substrate at the 1 L scale. After removing the resting cells and residual buffer a creamish white powder was obtained. Further, the purified product was analyzed by HPLC and showed a peak of pure butyrohydroxamic acid (RT: 2.273 min, Figure 8).

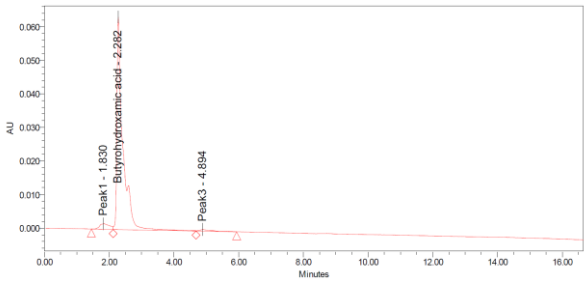


Figure 8. HPLC chromatogram of recovered butyrohydroxamic acid.

The HPLC analysis confirmed the yield of butyrohydroxamic acid as 100 mM (100%). In the 1 L batch mode reaction using resting cells, 10.23 g of butyrohydroxamic acid with 95% purity, 1.44 g/g/h catalytic productivity, and 2.273 g/L/h volumetric productivity were produced. Recombinant *Rhodococcus* sp. R312 amidase as an insoluble biocatalyst (10 g) produced 1 L of acetohydroxamic acid in 1 hr and 30 min with a bioconversion rate of 55 to 60% (mol/mol) (Fournand et al., 1997). Sharma et al. (2022), reported a 64% recovery rate for bench scale production of butyrohydroxamic acid with 96% purity. Singh et al. (2020), reported 83% recovery for the product vorinostat from the substrate N'-phenyloctanediamide. A bioconversion rate of 95% (mol/mol) has been reported for acetohydroxamic acid production by use of *Geobacillus pallidus* resting cells in 20 min at 50°C (Sharma et al., 2012). A 93% molar conversion yield at 1 L scale was obtained to produce acetohydroxamic (280 mM) acid by using the amidase of *Bacillus* sp. APB-6, while 0.26 M of acetohydroxamic acid was obtained in 80 min by using amidase of *G. pallidus* BTP-5X (Bhatia et al., 2013). Bhatia et al. (2015), obtained nicotinyl hydroxamic acid (190 mM) with 95% yield in 30 min at 1L scale, which was slightly higher than at a 50 mL scale. The catalytic productivity and volumetric productivity of the recovered nicotinyl hydroxamic acid were 13 g/h/dcm and 32 g/L/h, respectively (Boodhoo et al., 2022). Our data from bench scale 1L reaction reported higher molar conversion for the hydroxamic acid as compared to previous reports for acetohydroxamic acid and benzohydroxamic acid production. The bench scale reaction recovered 70% of the resting cells with 40%

residual amidotransferase activity. These cells were reused in a 50 mL reaction mixture and converted 34% of 100 mM butyramide to butyrohydroxamic acid. The standardized conditions with a high conversion rate at the bench scale can be further upscaled to industrial-level fermenters for the commercial production of butyrohydroxamic acid intended for various applications.

NMR spectrum of butyrohydroxamic acid

The OH proton is indicated by a singlet at 5.7009 (Figure 9).

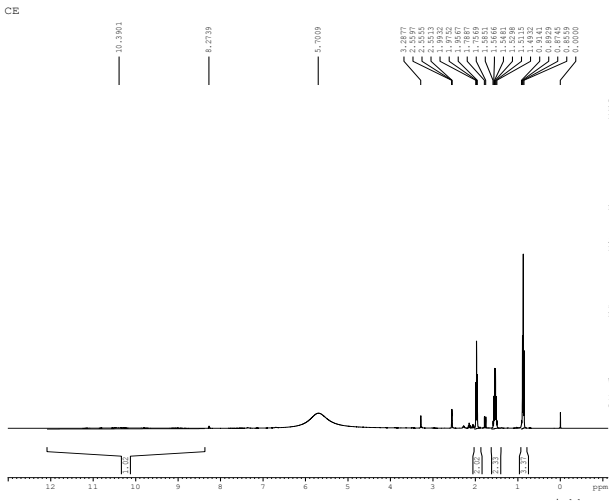


Figure 9. Proton NMR spectrum of butyrohydroxamic acid.

There is no coupling between the methylene protons and the hydroxyl protons. The methylene protons resonate at a lower field due to the adjacent oxygen atom and appear as a quartet at 0.875 ð for 2H. The methylene protons show a multiplet at 5.481 ð. The methyl protons appear as a triplet at 1.9752 ð for 3H. This data indicated the presence of butyrohydroxamic acid.

Conclusion

The present study showed the production of 100 mM butyrohydroxamic acid (100% molar conversion yield) on a 1 L bench scale. Also, bench scale reaction recovered 70% of the resting cells with 40% residual enzyme activity, which can be further reused in subsequent reactions.

This is the first report on the amidotransferase activity of the amidase from *Bacillus* sp. APB-6 with high butyramide tolerance. The optimized process against different parameters for the effective production of butyrohydroxamic acid by biotransformation can be upscaled and may find applications for various industrial bioprocess products, including anticancer compounds, antimicrobial compounds, and anti-inflammatory agents.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

Author Contributions

PK: experimentation, data collection; MP: data collection, and visualization, formal analysis and interpretation of the data original draft, review, and editing; AK: review and editing; DC: review and editing, supervising the work.

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