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PURIFICATION AND CHARACTERIZATION OF URICASE FOR RENEWABLE ENERGY

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Abstract
This research focused on harnessing uricase, a crucial enzyme in purine metabolism, from poultry waste for potential applications. Despite its absence in humans due to genetic mutations, uricase was successfully extracted from poultry waste with an initial activity of 3721.04 U/mL and a specific activity of 135.681 U/mg. Notably, the enzyme's activity increased to 9581.843 U/mL and specific activity to 534.641 U/mg after ammonium sulfate precipitation, resulting in decreased protein content. Further purification through ion exchange chromatography using DEAE cellulose and gel filtration with Sephadex G-150 demonstrated an enhanced activity of 3768.114 U/mL and specific activity of 1689.73 U/mg. The enzyme was purified 63-fold, with protein content reduced to 2.23 mg/mL. Optimal conditions for enzyme activity were observed at pH 8 and 37°C. The study delved into substrate and enzyme concentration effects, revealing maximum activity at 2.5 mg substrate (poultry waste) and 750µL enzyme concentrations. Additionally, the research explored the thermal stability of uricase, noting a maximum activity of 4095.18 U/mL after 20 minutes of incubation at elevated temperatures. The irreversible thermal denaturation decreased half-life from 660 to 149.4 min at 60°C compared to 37°C, with the enzyme showing positive ΔS*, ΔH*, and ΔG*. This work contributes valuable insights into utilizing uricase from poultry waste.

Keywords: ammonium sulphate ppt, fermentation, gel filtration chromatography, ion exchange chromatography, kinetics, thermal denaturation, uricase

Introduction:

Uricase, also known as urate oxidase (uox), plays a key role in purine metabolism, catalyzing the conversion of insoluble uric acid into highly soluble allantoin. The significance of uricase lies in its pivotal role in preventing the accumulation of uric acid, a compound associated with various health issues, particularly gout[1]. By facilitating the conversion of uric acid to more soluble forms, uricase contributes to the prevention of urate crystal formation, a hallmark of conditions like gout[1,2].

In the intricate metabolic pathways of purine degradation, uric acid emerges as a key end-product in the human body, resulting from the breakdown of purines during nucleic acid degradation. A series of enzymatic processes are catalyzed by uricase (UOX, EC1.7.3.3), which transforms uric acid into allantoin. Elevated UOX levels are essential for preserving homeostasis and processing uric acid, and they are present in birds, most vertebrates, certain primates, and reptiles[3,4]. Lack of uricase, which is associated with the inactivation of the hominoid Uox gene, can lead to hyperuricemia (HUA). This matter is particularly critical for individuals undergoing chemotherapy since it can exacerbate and result in a potentially fatal condition known as tumor lysis syndrome (TLS), which is typified by renal failure[2,5].

To enhance uricase production, a range of substrates have been added to the fermentation medium. Uric acid and n-alkane stand out among them. Because poultry waste is widely accessible, moderately affordable, and has the potential to create enzymes in a sustainable manner, we specifically chose it as the substrate for our study. The decision to utilize poultry waste is consistent with the broader goals of waste valorization and resource efficiency since it makes use of a resource that would otherwise be unused[6].

Numerous species, including microorganisms, plants, and mammals, are capable of producing or isolating uricase. Though traditionally obtained from plants and then animals, microbial fermentation is now widely acknowledged as the ideal technique for producing large quantities of enzymes. Microbial fermentation is a viable option for industrial applications because of its affordability, speed, and simplicity. Here, we demonstrate the potential of this approach for scalable and sustainable enzyme synthesis by using microbial fermentation to extract uricase from poultry feces[7,8].

There are two objectives for this study. Above all, we aim to demonstrate that uricase can be made from poultry manure, providing a sustainable means of producing enzymes that aligns with the most recent advancements in green biotechnology. Secondly, we attempt to use purification techniques that produce an exceptionally pure and useful form of uricase. Achieving these aims promotes not only the field of enzymology but also the broader goals of environmentally friendly enzymatic technology development, sustainable bioprocessing, and waste utilization.

Methodology:

The study aimed to optimize uricase yield using a systematic approach. Analytical-grade chemicals and reagents from Merck and Sigma were employed. A pure culture of gram-positive bacterium Bacillus subtilis from GC University, Faisalabad, Pakistan, was maintained on nutrient agar at 4°C. Sporulation medium, adjusted to desired pH, was solidified and incubated for 48 hours. Inoculum, prepared at pH 8, underwent autoclaving and 48-hour incubation on an orbital shaker[9].

Spore counting utilized a haemocytometer, adjusting concentration to 107-108 spores/mL. Bacillus subtilis spores were prepared in pH 8 nutrient medium through rotary shaking. Uricase production involved cultivating the strain in a basal medium with poultry waste as a nitrogen source, autoclaving, and inoculating with 2% spore suspension. Liquid-state fermentation in triplicate flasks, containing substrate, peptone, and yeast extract, occurred with autoclaving, inoculation, and 48-hour incubation at 30°C[10].

Enzyme assay at 293 nm measured absorbance decrease from urate oxidation. Buffers, HCl (1 M), NaOH (1 M), and uric acid solutions were prepared. Uric acid concentration was determined using a standard curve, and protein content was measured via the Biuret method. Uricase purification involved (NH4)2SO4 precipitation, gel filtration chromatography, and ion exchange chromatography using a DEAE cellulose column[9,11].

Kinetic and thermodynamic studies included optimum pH determination (2-10), optimum temperature analysis (20-80°C), and activation energy (Ea) determination through Arrhenius plot. Michaelis-Menten constants (Km and Vmax) were assessed using uricase assayed with 0.1-1% uric acid. Irreversible thermal denaturation involved incubation at 40-65°C, studying the 1st-order rate constant for activation energy. Thermodynamics of irreversible thermal inactivation calculated parameters using Eyring's equation. The systematic methodology optimized key parameters, emphasizing poultry waste utilization for sustainable substrate in uricase production from Bacillus subtilis[12].

Results and Discussion

The liquid-state fermentation (LSF) medium's optimization for the production of uricase from Bacillus subtilis yielded promising results under specific conditions (table 1).

Table 1: Optimum conditions of LSF medium to produce the maximum uricase

Sr. #	Optimum conditions	Result
1	pH	8
2	Temperature	37oC
3	Substrate level (poultry waste)	2.5 mg
4	Enzyme concentration	750µL

In this investigation, the substrate utilized to manufacture uricases was poultry waste. It was discovered that the optimal concentration of substrate for the fermentation medium to produce the maximum amount of enzyme activity was 2.5 mg. These findings are in line with previous research that stimulated uricase production using uric acid, a similar substrate. Studies by Green and Ronald (1955), Fattah and Hamad (2002), and others have demonstrated that uric acid is a helpful source of nitrogen for the production of uricase, which raises the enzyme's activity. It was shown that the choice of substrate significantly affects the enzyme's production. [11].

The researchers investigated the optimization of the fermentation medium's pH to achieve the highest uricase yield, revealing an ideal pH of 8.5. This finding is consistent with previous studies by Alamillo et al. (1991), Huang and colleagues (2004), Kai et al. (2008), and others demonstrating that uricase produced from various sources, such as Chlamydomonas reinhardtii and Bacillus subtilis, exhibited optimal activity at pH 8.5.

It is well known that choosing the optimal pH is crucial to optimizing the synthesis of enzymes since microbial metabolic activities are sensitive to pH changes [12].

The purification of uricase from poultry waste involved a series of steps, each contributing to the refinement of the enzyme. In the initial Crude Cell Lysate, the total protein content was measured at 27.42 mg/ml, with a corresponding total activity of 3721.04 units/mL. The specific activity at this stage was 135.681 U/mg, serving as the baseline for subsequent purification steps, denoted as a purification fold of 1. Subsequent Ammonium Sulfate precipitation at 40% saturation resulted in a significant reduction in total protein to 17.92 mg/ml, accompanied by a remarkable increase in total activity to 9581.843 units/mL. The specific activity surged to 534.641 U/mg, leading to a purification fold of 3.94. Comparable results were observed in the purification of uricase from Puccinia recondita, where ammonium sulfate precipitation was used to achieve a 9.5-fold purification with 0.028 U/mg of specific activity. The purification process continued with Ion Exchange Chromatography, resulting in a further decline in total protein to 6.03 mg/ml and a simultaneous increase in total activity to 5818.35 units/mL. The specific activity rose to 964.48 U/mg, achieving a purification fold of 7. Similar findings were observed in the purification of uricase from ox renal with 4.29 U/mg specific activity and 330-fold purified after receiving DEAE-Sephadex treatment [10]. The final purification step through Gel Filtration Chromatography demonstrated the highest refinement, with total protein content reduced to 2.23 mg/ml. This step resulted in a significant increase in total activity to 3768.114 units/mL, leading to an impressive specific activity of 1689.73 U/mg. The current purification fold has reached 12.45, emphasizing the effectiveness of the purification process in improving the concentration and activity of uricase derived from poultry waste (see tab 2). It was noted that the technique also successfully purified ox renal uricase, resulting in a specific activity of 50 U/mg using Sephadex G-200.

Table 2: Summary of Uricase Purification

Purification steps	Total protein (mg/ml)	Total activity (units/mL)	Specific activity (U/mg)	Purification fold
Crude Cell Lysate	27.42	3721.04	135.681	1
Ammonium Sulfate ppt. (40%)	17.92	9581.843	534.641	3.94
Ion Exchange Chromatography	6.03	5818.35	964.48	7.10
Gel Filtration Chromatography	2.23	3768.114	1689.73	12.45

The effectiveness of our purification methods was validated by comparing them with previous studies. Uricase from Pseudomonas aeruginosa was purified using DEAE-cellulose and Sephadex G-200, demonstrating a specific activity of 636.36 U/mg [10]. In contrast, combining fractions from DEAE cellulose and DE52 resulted in a 240-fold purification with a specific activity of 12 U/mg.

The purification folds were consistent with those reported in earlier studies, affirming the efficacy of the purification techniques employed in this investigation for purifying uricase from Bacillus subtilis. These findings confirm that the chosen purification methods are dependable and efficient in generating a highly concentrated and active uricase.

The kinetic and thermodynamic properties of uricase derived from Bacillus subtilis were systematically investigated to understand its enzymatic behavior. The maximum reaction rate (Vmax) was found to be 56.2 IU/mg.min, indicating the enzyme's catalytic efficiency. The Michaelis - Menten constant (Km) of 0.67 M suggests a high substrate affinity, while the activation energy (Ea) of 31.5 kJ/mol reflects the energy barrier for the enzymatic reaction. The optimum temperature for activity was determined to be 38°C, and the enzyme exhibited a melting temperature (Tm) of 60°C, indicating its stability. At a pH of 7.3, the enzyme showed optimal activity. The thermodynamic parameters revealed a Gibbs free energy of activation (ΔG*) of 93.24 kJ/mol, enthalpy of activation (ΔH*) of 28.73 kJ/mol, and entropy of activation (ΔS*) of -193 J/mol.K, highlighting the energy changes during the enzymatic process. Furthermore, the free energy of transition state binding (ΔG* E-T) was -116.61 kJ/mol, and the free energy of substrate binding (ΔG* E-S) was -10.94 kJ/mol, providing insights into the binding characteristics of the enzyme. Overall, these findings contribute to the comprehensive understanding of Bacillus subtilis-derived uricase and its potential applications in renewable energy.

Table 3: Kinetic and thermodynamic properties of *Bacillus subtilis-derived* uricases

Sr. #	Kinetic parameters	
	V_{max} (IU/.mg.min)	56.2±1.9
	K_m (M)	0.67 ± 0.013
	E_a (kJ/mol)	**31.5**
	Temperature optima (°C)	**38**
	T_m	60°C
	pH optima	7.3
	ΔG* (kJ/mol)	93.24
	ΔH*(kJ/mol)	28.73
	ΔS* (J/mol.K)	-193
	ΔG^*_{E-T} (kJ/mol)[b]	-116.61
	ΔG^*_{E-S} (kJ/ mol)[c]	-10.94

Conclusion: Bacillus subtilis-derived uricase, extracted from poultry waste, exhibits promising enzymatic properties, making it valuable for renewable energy applications and waste valorization initiatives.

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