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OPTIMIZATION OF ENZYME ACTIVITY AND FEATHER DEGRADATION UNDER VARIED EXPERIMENTAL CONDITIONS BY FUNGI *CHRYSOSPORIUM TROPICUM*

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Abstract

Chrysosporium tropicum, renowned for its distinctive morphological traits and potential for enzymatic activity, serves as a compelling subject for exploring keratinase production and growth optimization. This study delves into the determination of optimal conditions for keratinase production by Chrysosporium tropicum and investigates the correlation between its morphological characteristics and enzymatic activity. Through a series of experiments, the effects of incubation period, temperature, and substrate amount on keratinase production were evaluated. Keratinase activity was measured using a spectrophotometer, revealing significant variations across different conditions. Results indicate that the highest keratinase activity for Chrysosporium tropicum was achieved after 16 days of incubation at 25°C, with a substrate amount of 6g. Subsequent purification of the enzyme was conducted via dialysis and column chromatography, resulting in enhanced enzyme activity. The findings underscore the biotechnological potential of Chrysosporium tropicum in keratin degradation processes and contribute to our understanding of optimizing enzyme production for various applications.

Keywords: *Chrysosporium tropicum, Keratinase, Enzyme activity, Growth optimization, Morphological characteristics*

1. INTRODUCTION

Keratinophilic fungi are a specific category of fungi that has the exceptional capability to break down keratin, a resilient protein present in feathers, hairs, nails, and other outer layers of animals. The macroscopic characteristics of C. lunatus consist of a brown to black color, a hairy, velvety or fuzzy texture, and colonies that are loosely formed and develop fast on a potato dextrose agar medium.

Keratinophilic fungi inhabit keratin-rich materials and break them down into smaller molecular components. These fungus can be differentiated from others by their unique ability to consume keratinous proteins as sources of carbon and nitrogen.

These organisms often use proteins, peptides, and amino acids as sources of carbon, even when sugars are present (Kumar et al., 2021). These fungi are essential for breaking down keratinaceous materials found in nature, such as feather waste produced by chicken processing factories.

Gaining insight into the mechanisms that govern their adherence and breakdown of keratin is crucial for devising approaches to effectively handle keratin waste, such as feathers discarded during chicken processing, using biological methods such as fungal biodegradation. Keratin, in its natural form, is resistant to degradation by commonly occurring proteolytic enzymes such trypsin, pepsin, and papain.

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Microbial keratinase is an enzymatic substance with the ability to break down the tough, insoluble protein called keratin, which is present in feathers, hair, and wool. The resistance of this protein against destruction by proteolytic enzymes, such as trypsin, pepsin, and papain, is attributed to the specific composition and molecular conformation of the amino acids present in the keratin (Meenakshi et al., 2022).

Keratinases are enzymes that are synthesized by microorganisms in the presence of substrates that include keratin. Their main focus is on the disulfide (-S-S-) bonds that are found in keratin, a structural protein that is present in feathers, hair, and wool (Bockel et al., 1995).

Studies undertaken by multiple experts have discovered that the creation of keratinase in fungi, Streptomyces, and bacteria primarily takes place in alkaline pH conditions and at temperatures close to thermophilic levels. These enzymes have a broad range of substrates that they can break down, including keratin, fibrin, elastin, collagen, casein, bovine serum albumin, and gelatin (Noval et al., 1959; Mukhapadhayay et al., 1990; Dozie et al., 1994; Lin et al., 1995; Letourneau et al., 1998; Bressollier et al., 1999).

Over the years, multiple research have concentrated on extracting and characterizing keratinases from various microbial sources. These enzymes have been discovered in several types of fungi, bacteria, and actinomycetes. Microbial keratinases are highly important in multiple industries, such as tanneries, food processing, and waste treatment, due to their extensive range of uses and biochemical variety (Mini et al., 2016).

Keratinases possess a distinctive capacity to break down materials that are rich in keratin, which has resulted in their application in diverse biotechnological procedures that attempt to transform keratin waste into valuable products. Furthermore, the utilization of these enzymes in industries such as leather processing, food manufacturing, and environmental management underscores their significance in promoting sustainable and environmentally friendly practices.

Curvularia lunata is an interesting topic for studying keratinase enzyme activity and growth optimization due to its black, downy colonies and unique morphological traits, such as tall, unbranched conidiophores and olivaceous brown, smooth-walled conidia.

The objective of this study is to examine the most favorable circumstances for the synthesis of keratinase by Curvularia lunata and analyze the relationship between its physical attributes and enzymatic effectiveness. This study aims to expand our comprehension of the biotechnological capabilities of Curvularia lunata in keratin breakdown processes by investigating the impact of different environmental conditions on enzyme production and fungal development (Wang et al., 2022).

2. METHODOLOGY

For the production of enzymes, pre-cultured and identified fungal material of *Chrysosporium tropicum*, was utilized. Culture conditions were standardized using Potato Dextrose Agar (PDA) as the culture media, sterilized in an autoclave. The production media composition included yeast extract, di-potassium hydrogen phosphate, potassium dihydrogen orthophosphate, calcium chloride, and magnesium sulphate, with varying concentrations of glucose and peptone as carbon and nitrogen sources, respectively.

Additionally, the amount of chicken feathers, serving as the substrate, was varied accordingly. Glasswares utilized were of borosilicate quality, and chemicals such as glycine, sodium hydroxide, and tri-chloro acetic acid were employed in the process. Slants were prepared for long-term preservation of cultures, inoculated with *Chrysosporium* species, and incubated under specified conditions.

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The optimization of enzyme production parameters, including temperature, duration, and substrate amount, was conducted, followed by enzyme assay to assess the efficacy of the process.

2.1. Experimental design

Table 1: variables and their levels for central composite design

	PARAMETERS	-2	-1	0	+1	+2
X1	Duration (days)	4	8	12	16	20
X2	Temperature (°C)	20	25	30	35	40
X3	Feather amount (gms)	3	4	5	6	7
X4	Carbon source (gms)	1	2	3	4	5
X5	Nitrogen source (gms)	4	4.5	5	5.5	6

Table 2: central composite design arrangement

Serial no.	X1	X2	X3	X4	X5
1	0	0	2	2	-2
2	2	0	2	2	2
3	-1	0	-2	-1	0
4	-1	1	-2	1	0
5	2	0	-1	2	2
6	1	0	0	0	2
7	1	1	-2	2	2
8	2	1	0	2	2
9	0	0	1	-2	-1
10	-2	-2	0	0	0
11	-2	-2	-1	0	0
12	2	-2	-2	2	2
13	-2	-2	2	2	-2
14	-1	-2	2	2	-1
15	-1	-2	1	1	-2
16	2	2	-1	0	-1
17	-2	1	1	-1	-2
18	-2	0	-2	2	-2
19	-2	1	-1	2	-1
20	-1	2	2	-1	0
21	1	2	0	-2	2
22	0	2	-2	2	1
23	-2	2	1	0	-2
24	-2	2	2	1	0
25	-2	-1	1	2	-2
26	-1	-1	2	1	0
27	0	-1	-2	0	1
28	1	-1	-1	-2	0
29	1	-1	-2	1	2
30	2	-1	0	-1	-1

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2.2 Production of Enzyme

The production media, following the method outlined by Ramnani and Gupta (2004), was prepared for each experimental run. In each run, flasks were designated for different conditions: one flask served as a control with media and feathers but no inoculation, while others were inoculated with *Chrysosporium tropicum* -containing medium and no feathers, or with both medium and feathers. Inoculation was conducted using an inoculating needle. The flasks were then incubated at varying temperatures ranging from 20°C to 40°C for durations of 4, 8, 12, 16, and 20 days.

2.3 Preparation of Crude Enzyme Extract

Following incubation, the production medium containing the enzyme was filtered through pre-weighed filter paper, and the filterate was collected in small bottles. The filter paper contents were dried in an oven until moisture was completely removed and weighed to estimate the degradation of feathers. Centrifugation was performed at 5000rpm for 5 minutes, and the supernatant was collected as crude enzyme extract, which was then stored at 4°C for further use.

2.4 Enzyme Assay and Parameters Optimization

For enzyme assay and optimization of parameters, test tubes were labeled accordingly for each run. Crude enzyme from the respective flasks was added to the test tubes, along with 20mg of feathers. Control tubes received 3ml of 0.05M Glycine-NaOH buffer (pH 10), while other tubes received 4ml. Incubation was carried out at 60°C for 60 minutes, followed by the addition of 5% TCA to stop the reaction. After incubation at room temperature for 30 minutes, the contents were filtered, and the supernatant was collected for absorbance measurement at 280nm using a UV spectrophotometer.

2.5 Preparation of 0.05M Glycine-NaOH Buffer (pH 10)

A solution of 0.05M Glycine-NaOH buffer (pH 10) was prepared by dissolving 0.375g of Glycine in 100ml of distilled water and 0.2g of NaOH in 100ml of distilled water. Subsequently, 30ml of NaOH solution was added to 50ml of Glycine solution to achieve pH 10, and the final volume was adjusted to 200ml with distilled water.

2.6. Preparation of 5% TCA Solution

A 5% solution of tri-chloro acetic acid (TCA) was prepared by adding 5ml of TCA to 95ml of distilled water.

3. RESULTS

3.1. Degradation of feather by the fungi

	% of feather degradation(by weight)
Run no.	***Chrysosporiun tropicum***
1	12.0
2	42.3
3	56.0
4	74.0
5	62.5
6	96.0
7	87.4
8	99.8
9	15.0
10	66.0

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11	12.5
12	4.7
13	84.3
14	68.1
15	69.7
16	76.0
17	95.7
18	81.4
19	52.0
20	25.7
21	24.0
22	68.0
23	32.5
24	98.0
25	44.4
26	61.8
27	71.4
28	46.5
29	34.0
30	80.4

The table presents the results of an experiment measuring the percentage of feather degradation by weight for different runs involving Chrysosporium tropicum. Each row corresponds to a separate experimental run, with the percentage of feather degradation recorded for each run. For instance, the first run achieved 12.0% degradation, while the eighth run achieved a substantially higher degradation percentage of 99.8%. These results illustrate the varying effectiveness of Chrysosporium tropicum in degrading feathers across different experimental conditions or treatments. The table serves as a comprehensive summary of the extent of feather degradation achieved in each run, providing valuable insights for further analysis and experimentation in the field of feather degradation and bioremediation.

3.2. OPTIMIZATION OF CULTURAL CONDITIONS OF KERATINASE:

3.2.1 EFFECT OF TEMPERATURE ON KERATINASE PRODUCTION

Table 4: Effect of temperature on keratinase production

Fungi	TEMPERATURE (°C)				
	20	**25**	**30**	**35**	**40**
Chrysosporium sp.	42.8	60.2	94.6	76.0	62.0

The table illustrates the activity of the fungus Curvularia sp. at different temperatures (°C). At 30°C, the highest activity of 98.2% was observed, while activity decreased at both lower and higher temperatures, indicating an optimal temperature range for Curvularia sp. enzymatic activity. This table presents data on the growth temperature preferences of Chrysosporium sp., a type of fungus. The rows indicate different fungi species, while the columns represent different temperature conditions in degrees Celsius. The numerical values within the table represent the growth rates or levels of growth observed for Chrysosporium sp. at each temperature condition. For instance, at a temperature of 20°C, the growth rate of Chrysosporium sp. is recorded as 42.8. Similarly, at 25°C, the growth rate increases to 60.2, suggesting that Chrysosporium sp. prefers slightly higher temperatures for optimal growth. The data provided in the table allow for the comparison of growth rates of

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Chrysosporium sp. across different temperature conditions, providing insights into its temperature preferences and optimal growth conditions.

3.2.2. EFFECT OF INCUBATION TIME ON KERATINASE PRODUCTION

Table 5: Effect of incubation time on keratinase production

Fungi	Incubation period (Days)				
	4	8	12	16	20
Chrysosporium sp.	98.2	105.9	108.2	92.8	70.6

This table presents data on the incubation period (in days) of Chrysosporium sp., a type of fungus, under different time durations. The rows represent different fungal species, while the columns denote various incubation periods in days. The numerical values within the table indicate the incubation periods observed for Chrysosporium sp. at each time duration. For example, at a 4-day incubation period, Chrysosporium sp. exhibits an incubation period of 98.2 days, whereas at a 20-day incubation period, the observed period decreases to 70.6 days. The data provided in the table enable comparison of the incubation periods of Chrysosporium sp. across different time durations, providing insights into its growth characteristics and behavior under varying incubation conditions.

3.2.3. EFFECT OF AMOUNT OF SUBSTRATE ON KERATINASE PRODUCTION

Table 6: Effect of amount of substrate on keratinase production

Fungi	Amount of substrate (gm)				
	3	4	5	6	7
Chrysosporium sp.	64.7	82.3	98.0	70.2	52.9

This table presents data on the amount of substrate (in grams) used for the growth of Chrysosporium sp., a type of fungus, under different substrate quantities. The rows represent different fungal species, while the columns denote various amounts of substrate in grams. The numerical values within the table indicate the observed growth of *Chrysosporium sp.* at each substrate quantity. For example, when 3 grams of substrate are used, Chrysosporium sp. exhibits a growth of 64.7, whereas at 7 grams of substrate, the observed growth decreases to 52.9. The data provided in the table enable comparison of the growth of Chrysosporium sp. across different substrate quantities, providing insights into its growth characteristics and substrate requirements.

3.2.4. ENZYME ACTIVITY OF EXTRACTED KERATINASE ON THE SUBSTRATE

Table 7: Enzyme activity of extracted keratinase on the substrate

Serial no.	X1	X2	X3	X4	X5	% feather	Enzyme activity (purified
1	0	0	2	2	-2	12	116.1
2	2	0	2	2	2	42.3	99.2
3	-1	0	-2	-1	0	56	60.5
4	-1	1	-2	1	0	74	91.1
5	2	0	-1	2	2	62.5	75.5
6	1	0	0	0	2	96	58.2
7	1	1	-2	2	2	87.4	88.3
8	2	1	0	2	2	99.8	68.3
9	0	0	1	-2	-1	15	96.2
10	-2	-2	0	0	0	66	56.5
11	-2	-2	-1	0	0	12.5	88.3

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12	2	-2	-2	2	2	4.7	67
13	-2	-2	2	2	-2	84.3	95.7
14	-1	-2	2	2	-1	68.1	56
15	-1	-2	1	1	-2	69.7	71.2
16	2	2	-1	0	-1	76	88.8
17	-2	1	1	-1	-2	95.7	87.3
18	-2	0	-2	2	-2	81.4	66.4
19	-2	1	-1	2	-1	52	53.7
20	-1	2	2	-1	0	25.7	107.8
21	1	2	0	-2	2	24	72.3
22	0	2	-2	2	1	68	81.8
23	-2	2	1	0	-2	32.5	50.4
24	-2	2	2	1	0	98	56.8
25	-2	-1	1	2	-2	44.4	47.6
26	-1	-1	2	1	0	61.8	80.5
27	0	-1	-2	0	1	71.4	50.9
28	1	-1	-1	-2	0	46.5	56.3
29	1	-1	-2	1	2	34	174.8
30	2	-1	0	-1	-1	80.4	33.4

The table presents the results of an experimental study investigating the effects of different combinations of factors (X1 to X5) on both feather degradation percentage and enzyme activity (purified). Each row represents a unique experimental condition, identified by a serial number, with columns detailing the levels of the factors and the corresponding observed outcomes. For instance, in the first row, under the experimental condition where X3 and X4 are set to their highest levels (+2), the percentage of feather degradation was 12.0%, while the enzyme activity (purified) was 116.1. These results provide valuable insights into the relationship between the experimental factors and the outcomes of interest, aiding in the identification of optimal conditions for maximizing feather degradation and enzyme activity.

4. CONCLUSION AND DISCUSSION

In the production of keratinase from feather as substrate, the identified strains *Chrysosporium sp.* were used. The production media was inoculated for 4, 8, 12, 16 and 20 days and incubated at 30°C for the optimization of effect of incubation period on keratinase production. The enzyme activity was determined using a spectrophotometer. After production from above days, the enzyme assay showed that the maximum activity was produced after day 12 and 12 days i.e, 108.2 U/ml for *Chrysosporium sp.* which indicated that maximum enzyme was produced after day 8.

The production media was inoculated and incubated 20, 25, 30, 35.40°C for 12 days for the optimization of effect of temperature on keratinase production. The enzyme activity was determined using a spectrophotometer. After production from above days, the enzyme assay showed that the maximum activity was at 30°C i.e 94.2 U/ml for *Chrysosporium sp*.

The production media was inoculated and incubated 30°C for 12 days for the optimization of effect of amount of substrate on keratinase production. The enzyme activity was determined using a spectrophotometer. After production from above days, the enzyme assay showed that the maximum activity was at 5 gm 98.0 U/ml for *Chrysosporium sp*.

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In the next step, an experimental design was set up which included 30 runs. For each run cultural conditions such as temperature, amount of substrate, amount of carbon source, amount of nitrogen source and number of incubation days were maintained. The enzyme produced was filtered using filter paper and was then purified primarily by dialysis and secondary purification was done by column chromatography through sephadex G-57 gel.

The maximum enzyme activity for *Chrysosporium sp.* was observed in run number 29 i.e., 174.8 U//ml, which had the cultural conditions as: temperature 25°C, amount of substrate 4 g, amount of carbon source 1 g, amount of nitrogen source 5g and 16 days of incubation period. Thus maximum enzyme was produced after day 12.

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