





RESEARCH ARTICLE

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Anaerobic Process for Biohydrogen Production using Keratin Degraded Effluent

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Abstract

Fossil fuel energy depletion and removal of environmental wastes are big problem in many countries. Some of the wastes contain a considerable amount of protein and carbon compounds; little concentration is given to utilizing or recycling these wastes in a technological way for production of renewable energy. Feathers are generated in large amounts as waste at commercial poultry processing plant, reaching millions of tons per year worldwide. Feathers are rich in keratin protein. In this work, keratin was degraded and the degraded effluent was evaluated for biohydrogen production by anaerobic (dark fermentation) process. The keratin degrading microorganisms were isolated from the soil sample where the feather wastes were disposed using minimal media with prepared keratin powder. The isolates were named as KHAD1, KHAD2 and KHAD3. The isolates were identified as *Serratia marcescens* KHAD1 (MH422128), *Bacillus subtilis* KHAD2 (MH422129) and *Pseudomonas aeruginosa* KHAD3 (MH422009) based on their 16S rRNA sequencing. The isolates were screened for biodegradability of keratin protein and higher biodegrading potential microorganism such as *Pseudomonas aeruginosa* KHAD3. The optimization studies for biohydrogen production using isolates in keratin degraded effluent at various pH (1, 2, 4, 6, 8, 10 & 11). The maximum biohydrogen production was obtained at pH 11 (210 ml/L) by *Pseudomonas aeruginosa* KHAD3.

Keywords: Biohydrogen, Keratin biodegradation, poultry waste, chicken feather, Dark fermentation.

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INTRODUCTION

A large proportion of world energy needs are being covered by fossil fuels, which have led to an accelerated consumption of non renewable resources¹.

As the world population started to grow, mankind started for searching more effective means of energy production, turning towards the use of coal, oil and an even more energy efficient fossil fuel. Consequently the use of fossil fuels in order to cover the continuously increasing energy demands has resulted to the excessive accumulation of carbon dioxide bringing humanity to an imminent world energy crisis². The rapid depletion of fossil resources which is considered the main cause of global warming.

Nowadays, over exploitation of fossil fuels has brought mankind to a dead end from both an environmental and energy resource standpoint. Fossil fuels used as a major energy source for decades, caused undesirable emissions to the atmosphere leading serious air pollution problems. The indiscriminate use of fossil fuels and consumption of non renewable resources drive the current economy towards alternative energy sources. Considering global environmental impacts, such as green-house effect and resource recovery, there is a pressing need to develop non-polluting and renewable energy source³⁻⁵.

Hydrogen is the most promising alternative energy as it has a high energy yield of 142.35 KJ/g and high energy density of 122 KJ/g. It is the most abundant element which is clean, renewable fuel because the product of its combustion is only pure water and generates zero emissions when burned for energy recovery⁶.

Hydrogen is safer to handle than domestic natural gas. Hydrogen is now universally accepted as an environmentally safe, renewable energy resources and an ideal alternative to fossil fuels that does not contribute to the greenhouse. Presently, 40% hydrogen is produced from natural gas 30% from heavy oils and naphtha, 18% from coal and 4% from electrolysis and about 1% is produced from biomass. However, today, biological hydrogen production processes are becoming important mainly due to two reasons; utilization of renewable energy resources and usually operated at ambient temperature and atmospheric pressure⁷.

Biologically produced hydrogen is a valuable gas which is seen as a future energy carrier and therefore demand for hydrogen has increased considerably in recent years. Biological production of hydrogen provides a feasible means for the sustainable supply of hydrogen with low pollution and high efficiency, thereby being considered a promising way of producing hydrogen. Hydrogen production via biological routes provides a cost-effective and pollution free alternative to conventional thermal/chemical conversion of fossil fuels into hydrogen⁸. Biotechnology of hydrogen production has aroused a broad attention around the world as an environmentally friendly process.

Environmental wastes are found in large quantities in many countries. Although some of them contain a considerable amount of protein and various carbon compounds, little attention is given to utilizing or recycling these wastes in a technological way. Feathers are generated in large amounts as waste byproducts at commercial poultry processing plant, reaching millions of tons per year worldwide. Feathers are rich in keratin protein; keratins are stable, insoluble structural proteins found in the epidermis of vertebrates, its appendages such as feathers and hair. The α -helix or β -sheet keratin chains are tightly packed into a supercoiled polypeptide chain extensively cross-linked with disulphide bridges, hydrogen bonds and hydrophobic interactions, resulting in the mechanical stability of keratin and its recalcitrance to common proteolytic enzymes such as pepsin, trypsin and papain⁹.

However, despite its recalcitrance, keratin can be efficiently hydrolysed by keratinolytic enzymes produced by a multitude of bacteria and fungi and particularly representatives of the bacterial genus *Bacillus* appear as prominent keratinolytic micro-organisms¹⁰. Bacterial keratinolytic proteases show a potential to be utilized for feather bioconversion, enhancement of enzyme activities and increase in yields are required to make these suitable for industrial applications. Since feathers are almost pure keratin protein, feather wastes represent a potential alternative to more expensive dietary ingredients for animal feedstuffs. They require physical and chemical treatments to destroy certain aminoacids¹¹. Therefore, biodegradation of feather keratin by microorganisms represent an alternative

method to improve the nutritional value of feather waste and to prevent environmental contamination.

Keratinolytic activity assays and soluble protein determination using insoluble keratin or feather powder and culture supernatants was determined. Production of extracellular proteases by *Bacillus sp.* during growth on feather broth was evaluated by zymograms analysis. The keratin degraded effluent was analyzed for biohydrogen production as the effluent contains both the culture and degraded feathers. The present study was carried out to investigate the use of degraded feather waste for the biohydrogen production.

MATERIALS AND METHODS

Preparation of keratin substrate

Chicken feathers were collected from commercial poultry farm, Madurai. The feathers were washed thoroughly with water to remove the debris and then sundried for 3 days. The barbules present in the feathers were removed manually and oven dried at 75°C for 8 hours. After oven dried, the keratin materials were chopped into pieces and then milled using mixture grinder to obtain an approximate mesh particle size of 6 mm. The powder was used for further study.

Isolation and Identification of keratin degrading microorganisms

Keratin degrading microorganisms were isolated from the soil sample where the feather wastes were disposed. The soil sample was collected in sterile dark polythene bag and was transported to the laboratory. One gram of the soil was added to 9 ml of sterile saline blank and was serially diluted up to 10⁻⁹ dilution. 0.2 ml of the serially diluted samples was aseptically plated on minimal media containing keratin powder as a substrate. The plates were incubated at 37°C for 3 days¹².

The protein utilizing capability of the isolates was screened by growing on skim milk agar medium. The morphologically different microorganisms observed on minimal media containing keratin as substrate were individually streak plated on sterile skim milk agar medium plates. The plates were incubated at 37°C for 24 h and were observed for the clear zone around the colonies. The maximum clear zone producing organism was used for bacterial identification

studies.

The selected pure isolates were identified based on their microscopic, morpho-logical, biochemical characters¹³ and partial sequencing of their 16S rRNA. The isolation of DNA was done according to Janardhanan and Vincent¹⁴. The partial 16S rRNA was amplified according to Rochelle *et al.*¹⁵. Partial DNA obtained from PCR was sent for sequencing service. The sequences of the partial 16S rRNA were compared with the 16S rRNA sequence available in the public nucleotide databases at the National Center for Biotechnology Information (NCBI) by using its World Wide Web site (<http://www.ncbi.nlm.nih.gov>) and the BLAST (basic local alignment search tool) algorithm.

Degradation of keratin waste and Biohydrogen production

The Biodegradation of keratin waste and chicken feather by using the isolates was carried out as referred by Shawkey *et al.*¹⁶. The submerged cultivation using the isolates was performed on feather broth with Keratin powder or chicken feather as substrate. The chicken feather to be used in the experiment was washed with 0.1% Triton X100 and further washed with distilled water.

Sterile 50 ml of feather broth containing keratin powder or chicken feather with different pH (1, 2, 4, 6, 8, 10 and 11) was prepared and were aseptically inoculated with keratin degrading bacterial isolate.

To differentiate the level of keratin biodegradation, instead of using feather broth, minimal media¹² was used. One set of inoculated samples were incubated at 30°C in shaker for 7 days. Another set of inoculated samples were sealed with aluminum cap and nitrogen gas were sparged for 5 minutes and were incubated at room temperature for 7 days. After incubation, the samples were used for the analysis of biohydrogen, keratin biodegradation (weight loss%), physical and chemical Characterization (pH, ORP, Alkalinity, VFA, COD, BOD, Protein & Glucose).

Analysis of hydrogen production

At regular intervals, the gas produced in the headspace was analyzed using sterile glass syringe. The biohydrogen produced in regular intervals was expressed in ml.

Cumulative hydrogen production (ml/l) was obtained by using modified Gompertz

equation (Eq.1)^{17,18}; the Volumetric Hydrogen production rate (HPR) ($\text{ml l}^{-1} \text{h}^{-1}$) was calculated from cumulative hydrogen production (ml/l) divided by fermentation time (h). Hydrogen yield (HY) (mmol/mol glucose) was calculated as total molar amount of hydrogen divided by molaric amount of consumed glucose (as reducing sugar). The total molaric amount of hydrogen (mmol/l) was calculated using ideal gas law; total molaric amount of hydrogen (mmol/l) = Cumulative hydrogen production (ml/l) divided by RT. Where, $R=0.0821 \text{ atm K}^{-1} \text{ mol}^{-1}$ and $T=303 \text{ K}$.

$$H_{(t)} = P \cdot \exp \left\{ - \exp \left[\frac{R_m \cdot e}{P} (\lambda - t) + 1 \right] \right\} \dots \text{Eq. 1}$$

$H(t)$ is the cumulative hydrogen production (ml/l); P is the hydrogen production potential (ml/l); R_m is the maximum hydrogen production rate (ml/h); e is 2.71828; λ is the lag-phase time (h); t is time (h).

Analysis of keratin biodegradation

The degradation of feathers was evaluated by the dry weight of the keratinous waste remaining on culture broths during incubation. The culture broths were filtered through pre-weighed Whatmann No.1 filter paper and dried at 60°C until constant weight. Results were expressed as percentage of the initial weight (considered as 100%) of the respective keratinous waste.

Physical and Chemical Characterization

The fermented broth were analysed

for various physical and chemical characteristics such as pH, Oxidation-Reduction Potential (ORP), Volatile Fatty Acids (VFA), Alkalinity, Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), protein and reducing sugar by APHA¹⁹, Lowry et al.,²⁰ and Miller method²¹.

RESULTS AND DISCUSSION

Isolation and identification of Keratin degrading microorganisms

The keratin degrading microorganism was isolated from feather waste disposal area by using minimal medium containing keratin powder as substrate. Three morphologically different bacterial colonies were observed on minimal media containing keratin powder as substrate. The protein utilizing capability of the isolates screened by culturing the organisms on skim milk agar. Based on the morphological observation, the three isolates were named as KHAD1, KHAD2 and KHAD3. The partial sequences of these three keratin degrading microorganisms - 16S rRNA genes were determined. Based on the results of 16S rRNA partial gene sequence comparison with existing database in GenBank, the isolated bacterial strains were belonged to the genus *Serratia marcescens* KHAD1 (MH42-2128), *Bacillus subtilis* KHAD2 (MH422129) and *Pseudomonas aeruginosa* KHAD3 (MH422009).

Keratin degradation

In the feather broth medium keratin

Table 1. Feather Broth Medium with Keratin Powder: (Aerobic Degradation)

S. No.	Isolates	pH	Weight Loss (%)			
			Feather Broth Medium with Keratin Powder	Feather Broth Medium with chicken feather	Minimal Medium with Keratin Powder	Medium with chicken feather
1.	<i>Serratia marcescens</i> KHAD1	8	28	-	-	-
2.	<i>Bacillus subtilis</i> KHAD2	8	32	-	-	-
3.	<i>Pseudomonas aeruginosa</i> KHAD3	8	22	-	-	-
4.	<i>Serratia marcescens</i> KHAD1	10	69	1	13	-
5.	<i>Bacillus subtilis</i> KHAD2	10	63	2	11	-
6.	<i>Pseudomonas aeruginosa</i> KHAD3	10	58	1	15	-
7.	<i>Serratia marcescens</i> KHAD1	11	87	3	22	3
8.	<i>Bacillus subtilis</i> KHAD2	11	82	2	28	5
9.	<i>Pseudomonas aeruginosa</i> KHAD3	11	93	4	33	11

Table 2. Biohydrogen Production in feather broth medium with keratin as substrate

S. No.	Isolates	pH	ORP	Alkalinity (mg/L)	VFA (mg/L)	COD (mg/L)	BOD (mg/L)	Glucose (g/L)	Protein (g/L)	Cumulative hydrogen Production (ml/L)	Volumetric Hydrogen production rate (HPR) (ml l ⁻¹ h ⁻¹)	H ₂ Yield (mmol H ₂ /mmol glucose)	H ₂ evolved (ml/L)
1.	<i>Serratia marcescens</i> KHAD1	8	-023	3784	186	300	10.4	1.44	0.0083	-	-	-	-
2.	<i>Bacillus subtilis</i> KHAD2	8	-036	7150	805	300	4.32	14.73	0.0092	-	-	-	-
3.	<i>Pseudomonas aeruginosa</i> KHAD3	8	-058	1800	350	700	2.26	5.73	0.0174	-	-	-	-
4.	<i>Serratia marcescens</i> KHAD1	10	-017	17694	3081	180	8.03	26.57	0.0081	-	-	-	-
5.	<i>Bacillus subtilis</i> KHAD2	10	004	20494	963	180	8.24	27.48	0.0072	-	-	-	-
6.	<i>Pseudomonas aeruginosa</i> KHAD3	10	-027	11215	1294	180	8.01	17.7	0.0086	-	-	-	-
7.	<i>Serratia marcescens</i> KHAD1	11	109	29178	8754	700	2.7	29.94	0.0092	156.42	1.13	40.16	190
8.	<i>Bacillus subtilis</i> KHAD2	11	022	21080	4192	500	6.17	18.42	0.0054	-	-	-	-
9.	<i>Pseudomonas aeruginosa</i> KHAD3	11	214	36420	5754	1000	8.26	30.55	0.0125	172.88	1.25	44.39	210

Table 3. Biohydrogen production in feather broth medium with feather as substrate

S. No.	Isolates	pH	ORP	Alkalinity (mg/L)	VFA (mg/L)	COD (mg/L)	BOD (mg/L)	Glucose (g/L)	Protein (g/L)	Cumulative hydrogen Production (ml/L)	Volumetric Hydrogen production rate (HPR) (ml l ⁻¹ h ⁻¹)	H ₂ Yield (mmol H ₂ /mmol glucose)	H ₂ evolved (ml/L)
1.	<i>S. marcescens</i> KHAD1	10	-019	3871	172	200	2.03	8.07	0.0065	-	-	-	-
2.	<i>B. subtilis</i> KHAD2	10	-012	7120	792	200	3.09	5.86	0.0064	-	-	-	-
3.	<i>P. aeruginosa</i> KHAD3	10	-047	1400	315	600	8.23	5.62	0.0068	-	-	-	-
4.	<i>S. marcescens</i> KHAD1	11	218	6819	3042	400	5.98	19.49	0.0087	74.09	0.53	19.02	90
5.	<i>B. subtilis</i> KHAD2	11	024	29142	4186	0	6.15	10.42	0.0041	-	-	-	-
6.	<i>P. aeruginosa</i> KHAD3	11	002	11583	2018	400	4.14	28.20	0.0113	115.25	0.83	29.59	140

Table 4. Biohydrogen production in minimal medium with keratin as substrate

S. No.	Isolates	pH	ORP	Alkalinity (mg/L)	VFA (mg/L)	COD (mg/L)	BOD (mg/L)	Glucose (g/L)	Protein (g/L)	Cumulative hydrogen Production (ml/L)	Volumetric Hydrogen production rate (HPR) (ml l ⁻¹ h ⁻¹)	H ₂ Yield (mmol H ₂ /mmol glucose)	H ₂ evolved (ml/L)
1.	<i>S. marcescens</i> KHAD1	10	-021	3684	784	200	2.05	8.05	0.0128	-	-	-	-
2.	<i>B. subtilis</i> KHAD2	10	-025	7148	592	0	3.7	5.89	0.105	-	-	-	-
3.	<i>P. aeruginosa</i> KHAD3	10	-053	1782	253	600	8.22	5.43	0.0254	-	-	-	-
4.	<i>S. marcescens</i> KHAD1	11	003	48620	1082	400	5.94	9.47	0.0098	123.48	0.89	31.72	150
5.	<i>B. subtilis</i> KHAD2	11	108	58780	3492	0	6.17	13.57	0.0067	-	-	-	-
6.	<i>P. aeruginosa</i> KHAD3	11	-016	31750	953	400	4.14	8.16	0.0115	123.48	0.89	31.71	150

powder as substrate in different pH condition, the isolates were able to utilize keratin at pH 8, 10 and 11 and unable to utilize the substrate in pH 1, 2, 4 and 6. Among the three isolates, *Pseudomonas aeruginosa* KHAD3 shows the higher keratin degradability at pH 11 (93%). In the feather broth with feather as substrate, the isolates were able to utilize feather as substrate only at pH 10 and 11 at very less level when comparing it with feather broth medium containing keratin as substrate. In the minimal medium containing keratin powder as substrate, the organisms were able to degrade keratin at PH 8, 10 and 11 with a range of 11% to 33%. When comparing this result, feather broth medium with keratin powder lesser level of utilization was observed minimal medium containing keratin powder as substrate. In minimal media with feather as substrate, the organisms were able to utilize feather as substrate in higher pH of 11 with a range of 3 % to 11%. When comparing the keratin utilization in the form of powder or feather, the maximum substrate utilization was seen in feather broth medium with keratin powder at pH 11. In all the cultural conditions, the maximum substrate utilization was seen at pH 11. This suggested that, the isolates exhibit high range of substrate utilization at pH 11 (Table 1).

In anaerobic cultural condition, there was no significant substrate utilization in all pH except at pH 11 in the minimal media with keratin powder as substrate- *Serratia marcescens* KHAD1 (2%), *Bacillus subtilis* KHAD2 (8%), *Pseudomonas aeruginosa* KHAD3 (3%).

Biohydrogen

Biohydrogen production in feather broth medium with keratin powder as substrate at pH 11, the maximum of 210 ml/L medium hydrogen yield was obtained, produced by *Pseudomonas aeruginosa* KHAD3. At the same pH, the maximum of 190 ml/L ml medium hydrogen yield was obtained, by *Serratia marcescens* KHAD1 (Table 2). In chicken feather as substrate, the feather broth medium produced hydrogen yield at the maximum of 140 ml/L, at pH 11 by *Pseudomonas aeruginosa* KHAD3 (Table 3). In the minimal medium with keratin as substrate, at pH 11 maximum of 150 ml/L medium hydrogen yield was obtained by both the isolates *Pseudomonas aeruginosa* KHAD3 and *Serratia marcescens* KHAD1 (Table 4).

Table 5. Biohydrogen production in minimal medium with feather as substrate

S.No.	Isolates	pH	ORP	Alkalinity (mg/L)	VFA (mg/L)	COD (mg/L)	BOD (mg/L)	Glucose (g/L)	Protein (g/L)	Cumulative hydrogen Production (ml/L)	Volumetric Hydrogen production rate (HPR) (ml l ⁻¹ h ⁻¹)	H ₂ Yield (mmol H ₂ /mmol glucose)	H ₂ evolved (ml/L)
1.	<i>Serratia marcescens</i> KHAD1	11	-004	972	804	400	2.04	4.39	0.0738	-	-	-	-
2.	<i>Bacillus subtilis</i> KHAD2	11	-012	3498	563	200	4.19	8.24	0.106	-	-	-	-
3.	<i>Pseudomonas aeruginosa</i> KHAD3	11	-013	1653	318	800	8.26	5.54	0.0269	82.32	0.59	21.14	100

Minimal medium containing feather as substrate, produced maximum of 100 ml/L hydrogen yield, by *Pseudomonas aeruginosa* KHAD3 (Table 5). From this experiment, the maximum keratin degradation and biohydrogen production was seen in alkali condition (pH 11). Balint et al.,²² also reported the biohydrogen from keratin biowaste at pH 8.

CONCLUSION

Due to the rapid depletion of fossil fuels, emission of greenhouse gases causing global climate change and increasing oil price, there is an urgent demand for renewable, clean fuel alternatives for our future energy supply. Hydrogen, a regenerative and environmentally friendly fuel with high calorific value has attracted much attention by scientists. The hydrogen economy concept envisions that the future global energy demands will be mainly satisfied by hydrogen fuel instead of fossil fuels. The Hydrogen can be produced through different routes and from various substrates. Biological hydrogen production from a renewable biomass such as the organic fraction of waste or wastewater has considerable potential with respect to solving global environmental issues²³. Hydrogen energy will be defined as the usable heat or power derived from using hydrogen or a hydrogen rich fuel, as the single fuel source.

Molecular hydrogen derived from renewable sources is a potentially valuable alternative to fossil fuels. Two biological technologies to known to yield maximum rates of hydrogen production are (a) Photoproduction by purple photosynthetic bacteria requiring light energy and simple organic as reductants and (b) dark, fermentative hydrogen production acidogenic bacteria. But, both the methods have drawbacks.

Therefore, alternative method was needed. Concerning about the environmental wastes, feathers are produced in large quantities as a waste byproduct reaching millions of tons every year from poultry farms worldwide. Feathers are rich in keratin protein and due to the cross linking of disulphide bridges, hydrogen bonds and hydrophobic interactions, the feathers are not degraded by common proteolytic enzymes. Therefore, the feathers were degraded and the

degraded effluent was used for biohydrogen production.

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CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors have made substantial, direct and intellectual contribution to the work and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and the 16s rRNA sequences of Accession No. MH422128, MH422129 and MH422009 are available in NCBI-Nucleotide databases.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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