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The Novelty of Amino Acid Production From Feathers Degradation Employing Keratinase from *Streptomyces Venezuelae* AZ15

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Abstract:

Keratinases are enzymes belonging to the serine hydrolases group which are capable of degradation of keratin, an insoluble and fibrous structural protein widely cross-linked with hydrogen, disulfide, and hydrophobic bonds. Attempts to find new sources of enzymes and amino acids for fundamental knowledge of enzyme evolution, structure-function relationships, catalysis mechanisms of enzymes, and even for the identification of novel protein folds. In this study, seventy-nine samples were collected from different places in the University of Baghdad, and the best isolate for amino acid production by feathers degradation was by using *Streptomyces venezuelae* AZ15. The best fermentation system and the optimum culture conditions for amino acid production from feathers as substrate utilizing *S. venezuelae* AZ15 were elucidated. Using feathers as a substrate, the results showed that the submerged fermentation (SmF) system was the best to give the highest yield of amino acid. Furthermore, the highest yield of total amino acid was gained in feather broth supplemented with 2% glucose and 0.5% ammonium nitrate, with a pH of 6.0 at 37 °C for 8 days.

Keywords: Optimum conditions, Submerged fermentation, Solid state fermentation, keratinase.

الطريقة الحديثة في إنتاج الأحماض الأمينية من التحطيم الحيوي للريش باستخدام الكيراتيناز المنتج من بكتريا *Streptomyces venezuelae* AZ15

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الخلاصة

الكيراتينازات هي إنزيمات تنتمي إلى مجموعة سيرين المحللة القادرة على تحطيم بروتين الكيراتين ، وهو بروتين هيكلي ليفي وغير قابل للذوبان مرتبط على نطاق واسع بالهيدروجين وثاني كبريتيد والروابط الكارهة للماء. تعتبر محاولات العثور على مصادر جديدة للإنزيمات والأحماض الأمينية للمعرفة الأساسية بتطور الإنزيم ، وعلاقات التركيب والوظيفة ، وآليات تحفيز الإنزيمات ، وحتى لتحديد طيات البروتين الجديدة. في هذه الدراسة ، تم جمع 79 عينة تربية من أماكن مختلفة في جامعة بغداد وتم تحديد أفضل عزلة لإنتاج الأحماض الأمينية بفعل تحلل الريش باستخدام *Streptomyces venezuelae* AZ15. تم توضيح أفضل نظام تخمير وكذلك تحديد الظروف المثلى لإنتاج الأحماض الأمينية من الريش كمادة اساس وباستخدام بكتريا *S. venezuelae*

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AZ15 ، أظهرت النتائج أن نظام التخمر المغمور (SmF) هو الأفضل لإعطاء أعلى إنتاج من الأحماض الأمينية. علاوة على ذلك ، تم الحصول على أعلى محصول من الأحماض الأمينية في وسط الريش الزراعي المضاف إليه 2% جلوكوز و 0.5% نترات أمونيوم ، مع درجة الاس الهيدروجيني للوسط 6.0 عند 37 درجة مئوية لمدة 8 أيام.

1. Introduction:

Keratins are among the hardest-to-convert creature proteins. They are a significant part of proteins in hair, feathers, fleece, hooves, and nails. They are described as a firmly pressed structure in β -sheets and α -helices with a serious level of disulfide bonds [1]. They are insoluble, fibrous proteins that are widely cross-connected with disulfide, hydrogen and hydrophobic bonds. It is produced just within the sight of the keratin substrate. Indeed, this protein is obstinate to known proteolytic proteins: trypsin, pepsin, and papain [2]. For these reasons, researchers focus on the hydrolysis of this profoundly inflexible, unequivocally cross-connected primary polypeptide protein. Therefore, microbial keratinase has become biotechnologically significant. Keratinases are extracellular specialized proteolytic enzymes belonging to serine hydrolases enzymes that are equipped for the degradation of keratin [3]. These enzymes can be produced from *Bacillus* spp. and from other bacteria as well as they are produced from fungi including the species *Rhizomucor*, *Aspergillus*, *Penicillium*, and some of the dermatophytes including *Trichophyton mentagrophytes*, *T. rubrus*, *T. gallinae*, *Microsporum canis* and *M. gypseum*. keratinases are significant enzymes in some industrial steps such as the decomposition of feathers and hair in the poultry industry [4,5].

It is known that more than 20 naturally occurring amino acids function as the monomer building blocks of peptides, including proteins. Amino acids are chemical molecules with side chains (R groups) unique to each amino acid as well as amino (NH_3) and carboxyl group (COO^-) functional groups. Every amino acid contains the elements carbon (C), hydrogen (H), oxygen (O), and nitrogen (N). Additionally, the side chains of cysteine and methionine contain sulfur (S) [6]. Actinomycetes, especially *Streptomyces* in particular are known to produce a variety of proteases in culture medium. The serine proteases of *Streptomyces griseus* and *Streptomyces fradiae* are two of these proteases that have been structurally and enzymatically described. Numerous descriptions of the isolation and incomplete characterization of alkaline protease activity from other species of the genus *Streptomyces* have also been published. Extracellular proteases play a major role in these prokaryotic bacteria in the breakdown of big polypeptide substrates into smaller molecular entities that may then be absorbed by the cells [7]. These enzymes can break down the majority of nonstructural proteins and typically have low levels of substrate specificity.

The keratinases, a subset of the excreted proteinases that can breakdown the close packing of the protein chains in alpha-helix (α -keratin) or beta-sheet (β -keratin) structures, and the link of these structures by cysteine bridges. These enzymes are key factors in keratin's mechanical stability and resistance to microbial destruction. The biotechnological valorisation of keratin-containing wastes, such as feathers or hair as well as the leather industry is a prospective application for keratinases. In this industry, keratinases may be useful in the development of non-contaminating methods. A *Streptomyces* strain (strain K1-02) isolated from henhouse soil and cultured on feather meal as the only source of carbon and energy was found to have strong keratinolytic activities in the culture medium earlier [3,8,9, 10]. The goal of this study was to test amino acid production from the biodegradation of feathers chickens using *Streptomyces venezuelae* AZ15, and its use as a nitrogen source.

2. Materials and methods:

The soil samples were collected from a different area of the University of Baghdad, Iraq. The samples were aseptically transported to the laboratory and processed within six hours of collection. Nutrient agar and broth, Brain heart infusion agar and broth were purchased from Hi-media, India. Sodium acetate (CH₃COONa), Sodium hydroxide (NaOH), Ethanol 95%, and other materials were purchased from BDH, England.

2.1 Microorganism and inoculum preparation:

Seventy-nine local isolates were collected from different areas (were taken from the Biotechnology Department, College of Science\Baghdad University), and screened for keratinase production on keratin media. The local *S. venezuelae* AZ15 isolate was the best isolate for keratinase production, then the isolate of *S. venezuelae* AZ15 was subcultured and incubated at 37 °C for 48 hours. The isolates that were used throughout the trial were maintained on a nutrient agar slant, then inoculated slants were incubated at 37 °C for 48 hours; and at 4°C the slants were stored. To prepare the inoculum, the bacteria grown on a nutrient agar medium were transformed into the media broth. The inoculum was grown in a 250 mL flask containing 50 mL of feather broth (containing g/L pH 6: 20 feather, 5 yeast extract, 1 MgSO₄, and K₂HPO₄) at 37 °C on a rotary shaker at 120 rpm for 8 days [11].

2.2 Extraction and determination of amino acid:

Samples were collected from shake flasks and transferred to falcon tubes after filtration by filter paper and followed by centrifugation at 10000 rpm for 5 min. The amino acid was analyzed by spectrophotometric method determining absorbance at 570 nm using a standard curve of tyrosine employing 2% ninhydrin reagent [12]. The total amino acid was calculated by the equation:

Amino acid conce. µg/ml = Ab. At 570 nm/slope from a standard curve of tyrosine
Where Ab is the absorbance at 570 nm [12].

2.3 Types of fermentation system:

Two types of fermentation systems were used for the production of amino acid from the *S. venezuelae* AZ15 isolate, these systems were submerged fermentation (SmF) and solid-state fermentation (SSF). In submerged fermentation the medium used was described above, while the medium of solid-state fermentation was composed of:

A 250 mL flask containing feather 5 gm, 0.5 gm yeast extract, 0.1 gm MgSO₄, and 0.1 gm K₂HPO₄, then moisture 60 % of distilled water, the pH was adjusted to 6.0, then sterilized at 121°C for 15 minutes. This medium was for amino acid production by the *S. venezuelae* AZ15 isolate. Each flask containing production media was incubated with 2 ml of cell suspension (8×10⁶ cells/ml). In SmF group the flasks were incubated at 37 °C in the rotary shaking incubator (140 rpm) for 8 days. In SSF the production media were incubated at 37 °C for 8 days. The amino acid concentration was estimated [13].

2.3.1 Extraction of amino acid produced in solid-state fermentation:

After the end of SSF fermentation, the concentration of amino acid was measured, and the culture was first extracted using 25 mL of sodium acetate buffer (0.2 M pH 6). The mixtures were incubated in a rotating shaker for 2 hrs. at 37 °C with 140 rpm. Then the extract was filtrated by Whitman filter paper No. 1 for biomass removal. The amino acid concentration was then measured in µg/gm according to the following equation with some modifications:

Amino acid concentration ($\mu\text{g}/\text{gm}$ substrate) = Amino acid concentration ($\mu\text{g}/\text{mL}$) \times Total volume of extraction (mL)/Amount of substrate taken (gm). [13]

2.4 Optimization of cultural conditions for amino acid production:

Optimum conditions for amino acid production by the selected isolate were performed using SmF. The parameters were: Carbone sources, nitrogen sources, incubation temperature, pH, and incubation period.

2.4.1 Carbon sources:

Seven types of carbon sources were used to determine the optimal carbon source for amino acid production from feathers degradation by keratinase produced by *S. venezuelae* AZ15 isolate. These carbon sources including (20 gm/L): lactose, starch, fructose, sucrose, cellulose, maltose, and glucose, were added separately in different flasks containing feather broth. Then inoculation with 8×10^6 cells/ml and incubated at 37 °C for 8 days; amino acid concentration was estimated [13].

2.4.2 Nitrogen sources:

Eight nitrogen sources were used to determine the optimal nitrogen source for amino acid production from feathers degradation by *S. venezuelae* AZ15 isolate. These nitrogen sources including (5 gm/L): organic nitrogen such as yeast extract, urea, and casein as well as inorganic nitrogen sources such as ammonium nitrate, $\text{Ca}(\text{NO}_3)_2$, NH_4Cl , NaNO_2 , and $(\text{NH}_3)_2\text{SO}_4$, were added separately in different flasks containing feather broth plus glucose. Then inoculation with inoculum size 8×10^6 cells/ml and incubated at 37°C for 8 days; amino acid concentration was estimated [11].

2.4.3 Temperature:

To select the optimum temperature for amino acid production from *S. venezuelae* AZ15 isolate, flasks containing medium that was selected as an efficient medium for amino acid production were inoculated with 1 % inoculum size (8×10^6 cell/ml) and incubated at different temperatures of 25, 28, 30, 35, 37, 40, and 45 °C for 8 days, the; amino acid concentration was estimated [12].

2.4.4 Determination of pH:

The selective broth medium for degradation of feathers was prepared at different pHs 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, and 8, then flasks were inoculated with 1 % (8×10^6 cell/ml) of the *S. venezuelae* AZ15 isolate and incubated at 37 °C for 8 days, the amino acid concentration was estimated [13].

2.4.5 Incubation period:

The selective broth medium for degradation of feathers was inoculated with 8×10^6 cells/ml of the *S. venezuelae* AZ15 isolate and incubated at 37 °C for different incubation periods 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 days, the amino acid concentration was estimated [12].

3. Results and discussions:

3.1 Type of fermentation system:

The local *S. venezuelae* AZ15 isolate was the best isolate for amino acid production, then the isolate of *S. venezuelae* AZ15 was subcultured and incubated at 37 °C for 48 hours. Two types of fermentation systems were used for the production of amino acid from the degradation of feathers by keratinase from *S. venezuelae* AZ15, these systems were submerged and solid-state fermentation. The results of this experiment showed that the production of amino acid using *S. venezuelae* AZ15 isolate by solid-state fermentation (SSF) gave the lowest yield of

amino acid compared with the submerged fermentation (SmF) system which gave the highest concentration when using feathers as substrate. The concentration of amino acid produced by SmF was 223 $\mu\text{g/ml}$, whereas the SSF gave 205 $\mu\text{g/ml}$ (Figure 1).

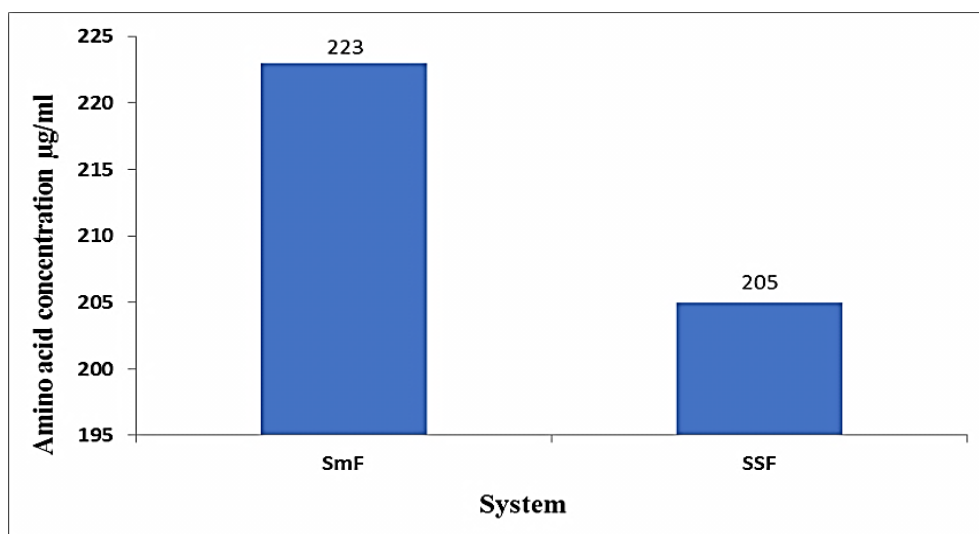


Figure 1: Production of amino acid from *S. venezuelae* AZ15 isolate by different systems.

Sahoo, *et.al.* [14], demonstrated that the submerged fermentation was a suitable system for the production of the keratinase enzyme from *Bacillus weihenstephanensis*, whereas the keratinase produced can degrade chicken feather keratin.

3.2 Effect of carbon source

The carbon sources' influence on bacterial growth and amino acid production from the degradation of chicken feathers were examined. *Streptomyces venezuelae* AZ15 was cultured for 8 days in a feather medium, in which different types of (20 g/L) carbon sources were added. As shown in Figure 2, among the 7 types of tested carbon sources, the outcomes indicated that *S. venezuelae* AZ15 potentially used various carbon sources for amino acid production from feathers degradation by the keratinase enzyme. The maximum amino acid was obtained in a medium containing glucose (807 $\mu\text{g/ml}$) while amino acid is low produced in other carbon sources. Although glucose is often a great supply of carbon for growth, it hindered the formation of a variety of primary and secondary metabolites. (15).

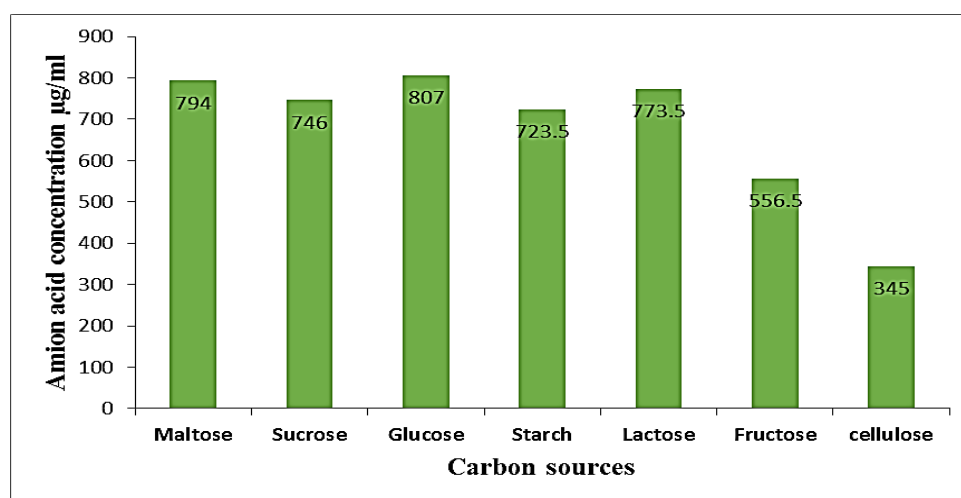


Figure 2: Effect of carbon source on the amino acid production by *S. venezuelae* AZ15 isolate using SmF, pH 6 incubation at 37 °C for 8 days.

Sahoo, *et.al.* [14], found that the best carbon source for maximum keratinase production from *S. weihenstephanensis* using feather media was cellulose. While Nnolim, *et.al.* [16], showed that the better carbon source for keratinase production from *Bacillus sp.* was xylose. Mohamad, *et.al.* [11], illustrated that the maximum production of the keratinase enzyme from *Pseudomonas sp.* was fructose as a carbon source when added to a feather medium. Several researchers used various sugars and observed an increase in keratinase production and clarified that the optimal carbon source for enzyme output varies depending on the microorganism. Increasing the enzyme leads to an increase in amino acid production from feathers.

3.3 Effect of nitrogen source:

Various nitrogen sources such as Ammonium nitrate, Yeast extract, Urea, Ca (NO₃)₂, Casein, NH₄Cl, NaNO₂, and (NH₃)₂SO₄ were supplemented separately for amino acid formation from feathers as substrate using *S. venezuelae* AZ15. It is widely known that the use of various nitrogen sources in fermentation has an impact on the growth of microorganisms and the generation of amino acids from feathers. The results showed that ammonium nitrate was the best nitrogen source for amino acid production after the biodegradation of feathers by keratinase enzymes. Ammonium nitrate, when used as a nitrogen source, yielded more amino acids than the other nitrogen sources, as seen in Figure 3. It has been claimed that a variety of amino acids found in organic nitrogen sources are crucial for the formation of primary metabolites. *S. venezuelae* AZ15 may manufacture the keratinase enzyme in the presence of various amino acids.

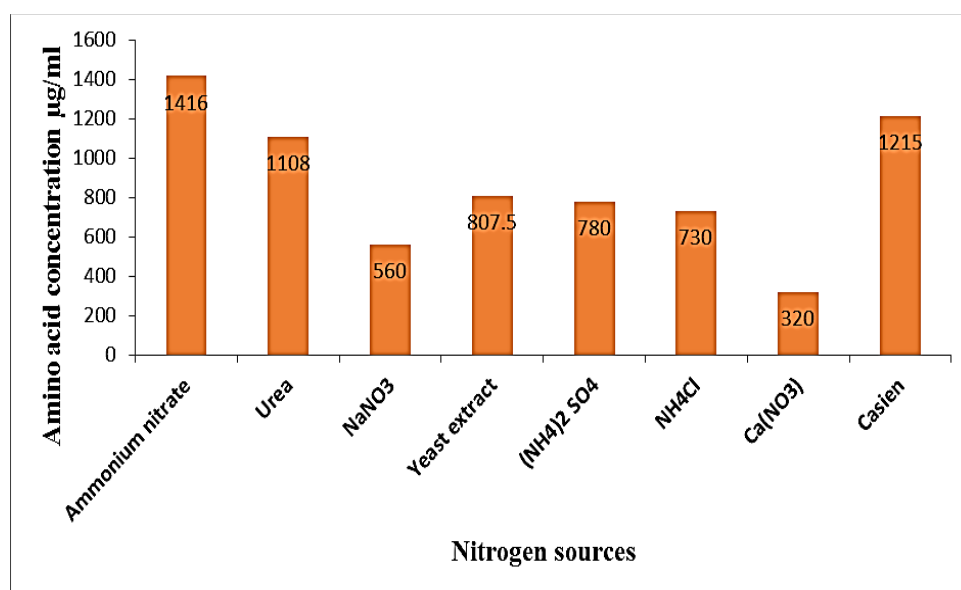


Figure 3: Effect of nitrogen source on amino acid production by *S. venezuelae* AZ15 using SmF, pH 6, incubation at 37 °C for 8 days.

Mohamad, *et.al.* [11], found that the maximum production of the keratinase enzyme from *Pseudomonas sp.* when peptone as a nitrogen source when added to the feather medium. While Nnolim, *et.al.*, [16], found that added nitrogen sources in the production media for keratinase production from *Bacillus sp.* led to a decrease in enzyme production. However, the presence of nitrogen in all forms is regarded as essential for the production of enzymes [17].

3.4 Effect of temperature:

The temperature has a definite impact on the bacteria's growth. However, the temperature had an impact on the generation of amino acids, with a rise in yield at 37 °C. The highest yield (1412 µg/ml) of amino acid was detected at 37 °C (Figure 4).

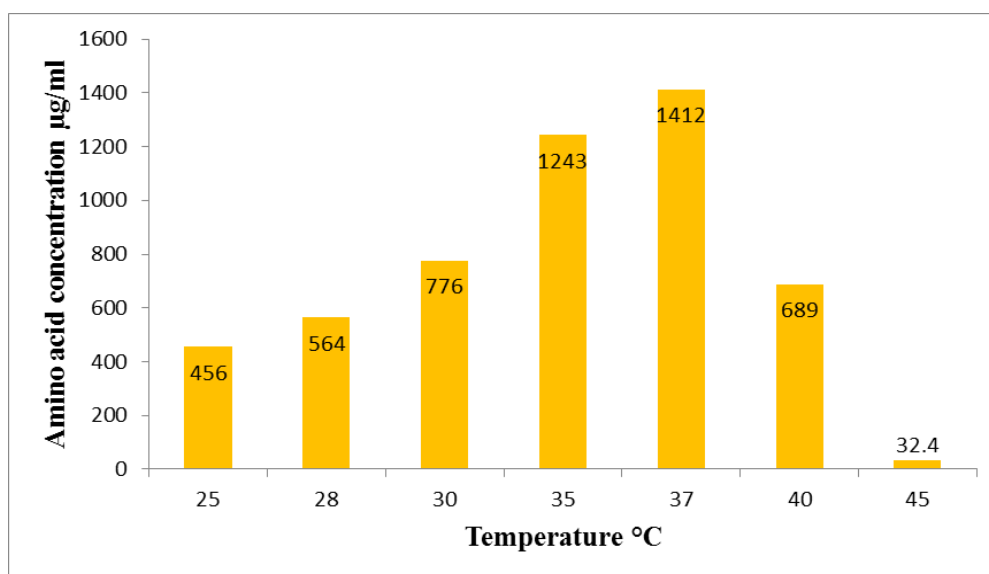


Figure 4: Effect of temperature on amino acid production by *S. venezuelae* AZ15 using SmF, pH 6, incubation for 8 days.

This finding is consistent with other microbes' synthesis of primary metabolites. One of the crucial factors that impact the effectiveness of the SmF system is temperature. Many previous works showed a close correlation between the synthesis of enzymes and bacterial growth, *S. venezuelae* AZ15 produces amino acids at a temperature that is also optimal for bacterial growth [18]. Singh, *et.al.*, [19], demonstrated that the maximum concentrations of the keratinase enzyme were obtained at temperatures of 40 °C, which were ideal for bacterial growth and the biodegradation of feathers to create amino acids during submerged fermentation.

3.5 Influence of pH:

The isolate of *S. venezuelae* AZ15 was grown on a feather medium with 20 g/L of glucose and 5 g/L of ammonium nitrate at a pH range of 3-8 for 8 days to examine the impact of the initial pH on bacterial growth and amino acid production. At pH 6.0, the maximum amino acid was obtained (1431.8 µg/ml). Figure 5 illustrates how pH affects the development of bacteria and the generation of amino acids. For the growth of *S. venezuelae* AZ15, a correlation between amino acid synthesis and starting pH was found (between pH 3 and 8). This outcome revealed that pH is important for the production of amino acids from degraded feathers. When the pH of the culture medium was set to 6.0, the best pH for amino acid production (1431.8 µg/ml) was noticed. In a related experiment, it was discovered that the production of keratinase which lead to the production of amino acids from the degradation of feathers during bacterial growth correlated with the proper concentration of hydrogen ions in the medium (pH 6). On the other hand, when the pH of the culture medium decreased or increased to below or above pH 6, bacterial growth was inhibited [11].

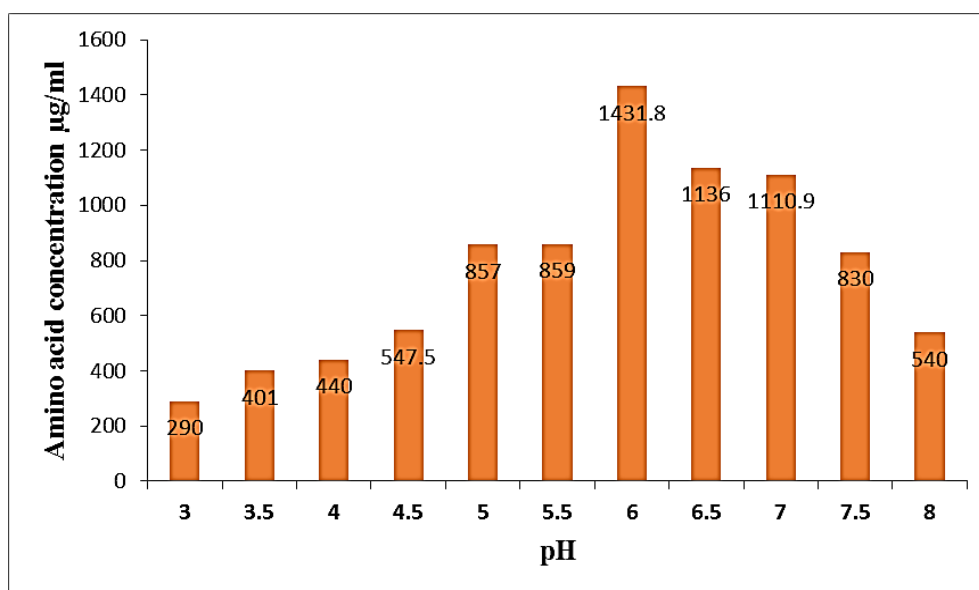


Figure 5: Effect of pH on amino acid yield employing *S. venezuelae* AZ15 using SmF, incubation at 37 °C for 8 days.

The critical factor in bio-mass accumulation and amino acid formation was the morphological difference of bacteria under different initial pH values. The ionic state of enzymes and substrates, the function of cell membranes, cell morphology and structure, the solubility of salts, the uptake of different feathers, and product biosynthesis may all be impacted by the pH of the medium. In general, cells can only expand within a certain pH range, and pH frequently influences the formation of metabolites as well [20]. Singh, *et.al.*, [19], found that the best pH for feather biodegradation and keratinase production from *Bacillus subtilis* was 7.0. Akhter, *et.al.*, [17], found that the best pH for feather biodegradation and keratinase production from *Bacillus subtilis* and *Pseudomonas* sp. were 7.0 and 10, respectively.

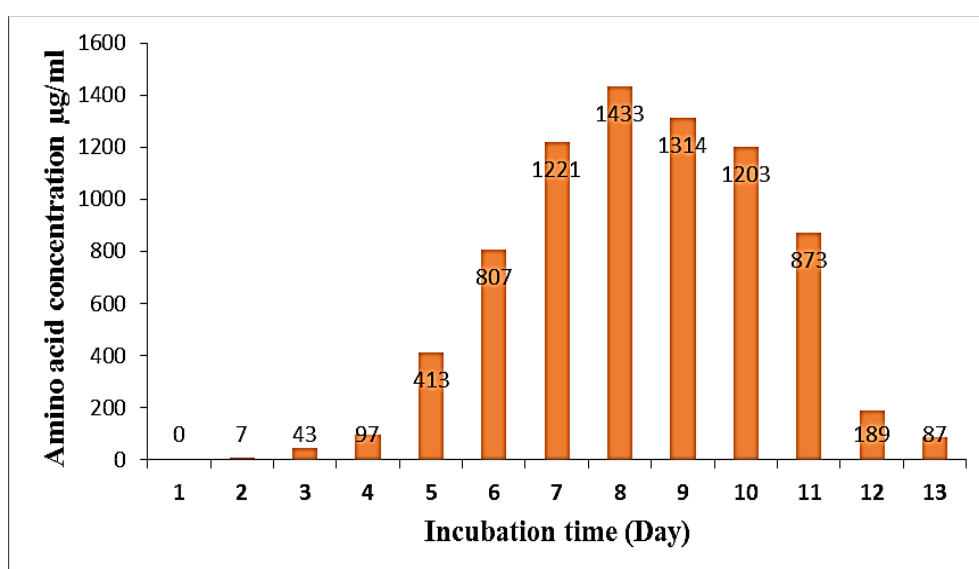


Figure 6: Effect of incubation time on amino acid production by *S. venezuelae* AZ15 using SmF, pH 6.0, incubation at 37 °C.

3.6 Effect of incubation time:

The optimization of feather biodegradation for amino acid production was initiated by incubation of the selected isolate with selected media at different time intervals (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13 days). To determine the better incubation time for amino acid production, several flasks were incubated for different time durations of one-day equal intervals. The maximum amino acid production was obtained in 8 days of incubation (1433 µg/ml) (Figure 6).

The feather biodegradation and amino acid production was decreased after 9 days. The decrease in amino acid production occurred as an outcome of reducing nutrient medium for isolate growth. Koutb, *et.al.* [12], illustrated that the better incubation time for feather biodegradation and amino acid production from feathers using *Aspergillus terreus* was 25 days. Singh, *et.al.* [19], found that the better incubation time for keratinase production by *Bacillus subtilis* is 72 hours, using a feather medium.

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Conflict of interest: The author declares no conflicts of interest.

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