Frontiers in Bioscience Elite

Front. Biosci. (Elite Ed) **2023**; 15(4): 27
https://doi.org/10.31083/j.fbe1504027

Original Research

Biological Ammonia Production via Anaerobic Fermentation of Soy Meal Protein

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Academic Editor: Yiannis Kourkoutas
Submitted: 9 September 2023 Revised: 19 October 2023 Accepted: 7 November 2023 Published: 5 December 2023

Abstract

Background: Conventional ammonia production methods, notably the energy-intensive Haber–Bosch process, are costly and contribute substantially to about 2% of the world's CO_2 emissions. This study focuses on the biological approach to convert protein to ammonia via hyper-ammonia-producing bacteria (HAB) fermentation. **Methods**: A consortium of ruminal microbes was employed in this work to ferment soybean meal protein under varying processing conditions. The parameters investigated included pH (7–11), inoculum concentrations (1–10%), substrate concentrations (5–20%), and fermentation time (0–168 h). **Results**: Optimal conditions for microbial growth and biological ammonia production were observed at pH 7, fermentation duration of 72 h, inoculum concentration of 10%, and substrate concentration of 10%. ~8000 mg/L biological ammonia was produced following HAB fermentation. **Conclusions**: By leveraging the capabilities of rumen HAB, this study contributes to the ongoing efforts to develop environmentally friendly processes for ammonia production that will mitigate both economic and environmental concerns associated with traditional methods.

Keywords: soy meal protein isolate (SMPI); fermentation; pH; alkalinity; biological ammonia; hyper-ammonia-producing bacteria

1. Introduction

The production and utilization of ammonia have reached unparalleled levels, making it a crucial chemical worldwide [1,2]. This indispensability stems from the wide-ranging applications of ammonia in industries such as textiles [3–5], oil and latex [6–9], and maritime [10,11]. However, its most critical role lies in the fertilizer industry, where ammonia accounts for over 80% of global food production, thereby fulfilling the escalating global demand for food [12–16]. With an estimated annual economic turnover of approximately 100 billion USD, ammonia's global production reached around 150 million metric tons (MT) in 2021 [1].

The recent global energy crisis, which has been mostly impacted by the Russian–Ukrainian conflict, has triggered surging energy costs and scarcity, resulting in a 25% reduction in global ammonia fertilizer production [17,18]. Consequently, ammonia scarcity is projected to increase [19]. Although the Haber–Bosch process—the conventional method of ammonia production is efficient [20,21]—it is challenged by high energy demand and costs [20,22]. Moreover, the Haber–Bosch process seems non-sustainable because it contributes to approximately 2% of the global CO_2 emissions, thereby exacerbating greenhouse gas emissions [1,23–25]. As a result, the search for innovative, renewable, sustainable, and eco-friendly technologies for ammonia production has become imperative.

Many approaches have emerged as potential solutions to address cost, energy demand, sustainability, and greenhouse gas emission issues in ammonia production [1]. Electrochemical synthesis, photoelectrochemical methods, and electromagnetic synthesis have been reported as potential alternatives for ammonia production [26–30]. However, each of these methods has its own limitations, as recently reported in our review article entitled "Trends in biological ammonia production" [31]. Conversely, biological technologies encompass biological nitrogen fixation (BNF) and microbial fermentation of nitrogen-containing biomass [1]. BNF involves the use of nitrogen-fixing microbes, such as bacteria and blue–green algae, to convert atmospheric nitrogen into usable forms [32–34]. While BNF can produce a substantial amount of ammonia annually, up to 1.8×10^{14} tons, it faces challenges such as the requirement for a large amount of adenosine triphosphate (ATP), specific proteins, and an oxygen-sensitive nitrogenase, which are scarce resources [32–35]. Furthermore, most of the involved microbes assimilate ammonia intracellularly, thereby limiting ammonia availability in the ecological system [35]. Although various microbial engineering approaches have been employed to overcome this ammonia assimilation issue, the volume of ammonia produced is currently insufficient for industrial feasibility [1].

Another emerging area in biological ammonia production is ruminal microbial fermentation of biomass [1]. The rumen of ruminants harbors specialized microbes known as

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hyper-ammonia-producing bacteria (HAB) [36–39], which efficiently convert dietary proteins to surplus ammonia [40–43]. These HAB consortiums include *Selenomonas ruminantium*, *Peptostreptococcus elsdenii*, *Bacteroides ruminicola*, *Escherichia coli*, and *Klebsiella quasivariicola* [1,35,44], which possess the ability to utilize nitrogen-containing substrates as their sole nutrient source, enabling simultaneous growth and substantial ammonia production [1,45,46]. Optimal growth conditions for HAB include a temperature range of 38 °C to 42 °C, a pH level between 5.5 and 7, and anaerobic conditions [1,42]. Through fermentation, they hydrolyze proteins into amino acids, which are further metabolized to produce protein and/or ammonia through ammonification [47–49].

However, the current utilization of dietary proteins by HAB in the rumen is an inefficient process. While dietary protein is crucial for animal products, the ammonia produced is absorbed from the rumen, metabolized, and excreted in the urine, resulting in inefficient use of dietary proteins and environmental nitrogen pollution [50]. A few attempts have been made to employ HAB in the fermentation of dietary proteins to produce biological ammonia. However, in a study investigating the bioconversion of different protein sources, soy protein isolate (SPI) was found to be the most effective substrate for bio ammonium production [1,51]. The efficiency of fermentation is influenced by several factors, including pH, inoculum concentration, substrate concentration, and alkalinity. Thus, understanding the influence of these parameters on HAB fermentation is crucial [52] in enhancing the yield of biological ammonia.

2. Materials and Methods

2.1 Chemicals

Analytical grade chemicals and media components were purchased from Sigma-Aldrich Chemicals, Saint Louis, MO, USA.

2.2 Preparation of Soy Meal Protein Isolate (SMPI)

Soy meal protein isolate from soybean meal was extracted using a solvent extraction method, according to [53]. Briefly, soybean flour of particle size 0.425–1 mm was soaked in 0.5% ammonium hydroxide (NH_4OH) solution at 1:10 sample-to-solvent and a temperature of 55 °C, with continuous shaking for 12 h. The mixture was centrifuged at 10,000 g, 25 °C for 10 min to remove any insoluble material. The supernatant containing crude protein extracts was collected and its pH was adjusted to 4.5–5. The protein isolate was allowed to sediment overnight before the sediment was collected, washed twice with distilled water, and centrifuged at 10,000g and 25 °C for 10 min. Protein was collected into an aluminum plate and dried at 60 °C for 12 h. The moisture content in the protein isolate was assessed and considered throughout the hydrolysis process.

2.3 Hydrolysis of Soy Meal Protein Isolate

The extracted SMPI was hydrolyzed according to [1]. Briefly, about 4 g SMPI was dispersed into 100 mL distilled water. Then, the pH of the solution was adjusted to 2.0 using 1 M HCl. Following pH adjustment, the solution was incubated in a water bath at 37 °C for 30 min, after which pepsin (0.3% w/w) was added. The SMPI–enzyme mixture was incubated in a water bath for 10 h followed by pH adjustment to 7.0 using a 2 M NaOH solution to halt the hydrolysis process. Subsequently, the enzyme digests were centrifuged at 5000 g for 3 min to eliminate any insoluble residues. Then, the resulting supernatant was appropriately diluted with a basal medium to produce the hydrolysates used in the subsequent fermentation experiments.

2.4 Isolation, Enrichment, and Selection of Appropriate Inoculum Concentration

Approximately 40 mL of rumen fluid containing microbial consortium was collected from three fistulated dairy cows at the North Dakota State University (NDSU) Animal Nutrition and Physiology Center. The fluid that was initially stored in 50 mL plastic screw-cap tubes was immediately transferred into an incubation chamber (39 °C) to maintain the microbial ruminal environment prior to its use. The HAB isolation and enrichment medium was prepared following the method described by Adeniyi *et al*. [1]. The raft in the ruminal content was allowed to settle and the resulting ruminal fluid was diluted 1:100 to obtain an optical density (OD) of ~0.05 before inoculation into a casamino acid medium 1.5% (w/v). The enrichment culture, which was set up in a BACTRONEZ Anaerobic Chamber, Sheldon Manufacturing Inc. (Cornelius, NC, USA) was incubated in a shaker set at 39 °C and 130 rpm. The culture was routinely transferred into fresh casamino-containing medium every 24 h to maintain a high HAB population. To determine the optimum inoculant concentration for fermentation, the effect of varying inoculant concentrations (1%, 2.5%, 5%, and 10%) on biological ammonia production was investigated at different time intervals.

2.5 Fermentation Conditions and Ammonia Production

The fermentation medium consisted (g/L) of K_2HPO_4, 0.292; KH_2PO_4, 0.292; Na_2SO_4, 0.480; NaCl, 0.10; $CaCl_2.2H_2O$, 0.064; Na_2CO_3, 4.0; yeast extract, 0.50, with the pH initially adjusted to 6.5–6.6 [54]. As described by Adeniyi *et al*. [1], 250 mL Erlenmeyer flasks containing 100 mL mixture of SMPI hydrolysate (4% w/v) and fermentation media were inoculated with 1 mL of inoculant and incubated at 39 °C, 130 rpm for different time ranges (24–168 h), to investigate substrate utilization by ruminal HAB for microbial growth and ammonia production. Prior to inoculation, SMPI hydrolysate–fermentation mixture media were autoclaved at 120 °C for 1 h. At 24 h fermentation intervals, samples were taken and filtered through Whatman qualitative filter paper,

Grade 1. The first sampling was conducted earlier—12 h after fermentation—to monitor and adjust the fermentation process in its initial stages and assess the performance of the microbes.

2.6 Determination of Microbial Growth

The concentration of cell biomass was determined through the utilization of the optical density (OD) method. At various time intervals, the OD of the fermentation media was measured at 600 nm using a UV-Vis Spectrophotometer (Varian Cary 4000, SpectraLab Scientific Incorporation, Markham, ON, Canada).

2.7 Determination of Optimum Substrate Concentration

The effect of varying substrate concentrations (2.5%, 5%, 10%, 15%, and 20%) on biological ammonia production was assessed and the substrate concentration that produced the highest amount of biological ammonia was selected.

2.8 Alkalinity Strength Test

Rumen bacteria maintain their intracellular pH close to neutral [55,56]. To study the effect of pH on microbial growth, a fermentation medium containing 1.5% soybean meal protein hydrolysate was agitated at pH levels ranging from 7 to 11. Fermentation was carried out using 1 mL of inoculant at 39 °C and 130 rpm for different time intervals (12, 24, 36, 48, 72, 96, 120, 144, and 168 h).

2.9 Ammonia Quantification

The quantification of ammonia produced was determined by colorimetric assay using the Nitrogen–Ammonia reagent kit (Hach Company, Catalog No. 50-199-6332, Loveland, CO, USA). A precisely measured 10 µL sample of the filtered fermentation product was diluted to 1000 µL in distilled water, resulting in an X100 dilution. The reagents for the test kit, ammonium cyanurate, and ammonium salicylate, were added to approximately 20 mL of test fluid and mixed thoroughly with 100 µL of the diluted sample to create the working reagent solution. A control blank solution was created by combining 100 µL of distilled water with the test kit reagents. The Hach DR3900 (Hach Company, Loveland, CO, USA) benchtop spectrophotometer was calibrated by utilizing the blank solution to establish a zero reference. Then, the prepared working reagent solution and samples were loaded into the spectrophotometer (at 655 nm) for analysis using the NH_3-N program.

2.10 Data Analysis

All data were collected in triplicate and subjected to statistical analysis using the standard error bars to measure statistical significance.

3. Results and Discussion

3.1 Effect of pH and Alkalinity on HAB Growth

The alkalinity strength of microbes is a crucial determinant of how they can resist changes in their environmental pH level. Conversely, optimum pH is the pH at which microbes exhibit maximum activity [57]. The alkaline pH levels were investigated because the fermentation process produces ammonia, which causes the pH of the medium to naturally increase as it is produced. Although alkaline pH levels can improve protein solubility and bioavailability, it is currently unknown whether this would increase the proliferation of the HAB. The present study investigated the alkalinity strength and optimum pH for microbes during soy protein fermentation at varying pH levels, to understand the effect of pH on HAB growth and determine optimum fermentation pH level. Thus, we conducted an experiment to monitor HAB growth profiles at different pH concentrations (7, 8, 9, 10, and 11) over time ranges of 12, 24, 36, 48, 72, 96, 120, 144, and 168 h.

The results show that microbial growths vary across different pH levels. As expected, at all pH concentrations, the microbes exhibited sigmoidal growth curves with varying optical densities and patterns at their exponential, stationary, and death phases [58] (Fig. 1). After the initial 12 h lag phase, a major growth transition occurs that depicts the onset of the log phase. During the log phase, the growth rate experiences a sharp acceleration, leading to exponential proliferation of the microbial population, indicating a state of high reproduction [59]. As shown in Fig. 1, microbial growth at a pH of 7 exhibits a particularly favorable environment for this robust proliferation during the log phase. Therefore, a pH of 7 enhances the exponential growth of the population.

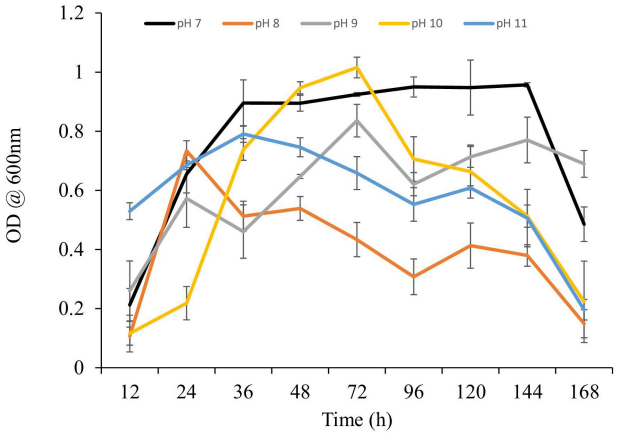

Fig. 1. HAB microbial growth curve at varying pH levels (7–11) and time (12–168 h). OD, optical density; HAB, hyper-ammonia-producing bacteria.

The stationary phase follows the log phase and represents a zero net specific growth rate, which is a crucial

stage that is marked by the expression of genes essential for organism survival [60]. Thus, the survival and progression of cells are closely tied to the length of their stationary phase [60,61]. Out of all the pH conditions, the stationary phase growth pattern at pH 7 was the longest, spanning from 36 to 144 h. Therefore, an environmental pH of 7 provides stability and offers support for the HAB population to consume cellular reserves, while also sustaining their persistence. This result is consistent with previous studies on rumen bacteria, which suggest that rumen bacteria tend to thrive well at a near-neutral pH [55,56,62]. The overall observation suggests that a pH of 7 is the optimum concentration for HAB proliferation during soy meal protein fermentation.

Furthermore, an optimum pH of 7, which was observed during HAB soy meal protein fermentation, can be understood from the perspective of cellular energetics. Bacteria can effectively modulate proton and ATP production levels by leveraging the interplay between cellular bioenergetics (electrochemical proton gradient and ATP synthesis) and the physiochemical charge regulation effect that occurs on cell surfaces [63]. Studies have shown that cellular pH has a significant impact on cellular processes, including microbial growth for optimal energy generation [64,65]. Cells exhibit an active and complex regulation system to maintain energy homeostasis by carefully controlling their internal pH through the precise release or consumption of protons [65,66]. The surrounding medium, where cells reside, acts as a reservoir of protons, its role depending on the presence of specific substrates and their interactions [65]. These proton exchange dynamics become interesting when studying less explored organisms, such as hyper-ammonia-producing bacteria since they not only possess the ability to produce ammonia but may also rely on extracellular electron transfer for their ammonia production mechanism. Given the constant production of ammonium, it is possible for the microbes to assimilate them as a nutrient and energy source [31], thereby releasing protons and causing a reduction in pH [67]. A rise in pH is also possible following the attachment of bacteria to surfaces containing positively charged amino groups [64]. This pH elevation is significant enough to cause a decrease in ATP levels. Decreased ATP levels undermine the capability of proton pumps to actively remove protons, thereby causing an accumulation of protons within the cell. This accumulation can also result in a decrease in pH, which makes the cell more acidic. However, the fermentation process aids in maintaining a near-neutral medium, as the generated ammonium offsets the liberated protons. The amount of ammonium utilized to neutralize the released protons is also proportional to microbial growth [68].

Studies show that different microbes have different mechanisms for surviving pH variations. Thus, the ability of microbes to survive and thrive under varying pH levels depends on their capacity to maintain internal pH homeostasis, adapt to changes in external pH levels, and utilize specific pH-related metabolic processes [69–74]. For instance, alkaliphilic bacteria, such as *B. pseudofirmus* OF4 in organic amine-rich media are challenged by the potential for cytoplasmic accumulation of ammonium at the cost of cytoplasmic protons [74]. This cytoplasmic ammonium accumulation poses a risk to their pH homeostatic mechanisms, potentially disrupting intracellular pH balance. In response, the alkaliphilic bacteria have evolved specific adaptations, such as the ammonium efflux system that supports bacterial growth by expelling ammonium from its cytoplasm [74]. In contrast, a study on the effect of high ammonium concentrations on neutralophilic bacteria suggests that high ammonium concentrations had no major repercussions on their overall viability and growth [74,75]. The effect of high alkalinity has also been investigated in Gram-negative and Gram-positive bacteria, such as *E coli* and *E subtilis*, respectively [75]. Results show that no negative effect of elevated ammonium concentrations was observed, except for some growth retardation, which was attributed to osmotic and ionic effects. A plausible reason for their survival in higher pH concentrations is the presence of antiporters and higher levels of transporters and pro-proton capture and retention enzymes [61].

In most fermentation reactions, the pH of the media is controlled to a point that is amiable to microbial proliferation and target product formation. Ammonia, being an amphoteric compound, has the potential to either elevate or decrease the pH of a medium [76]. During biological ammonia production via HAB fermentation, the direction of pH change is unknown. Consequently, we monitor pH fluctuations throughout the biological ammonia production process. Fig. 2 shows the alkalinity strength of microbes for a given time range (12–168 h).

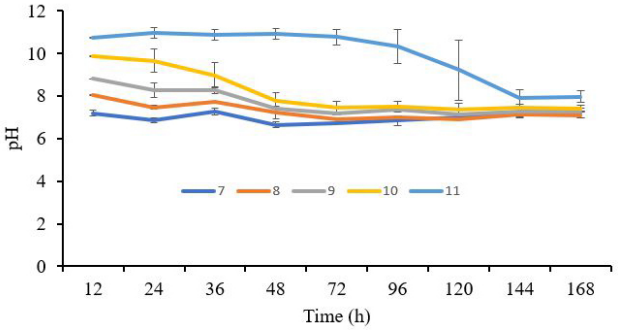

Fig. 2. Alkalinity strength by HAB for a given time range (12–168 h).

The result shows that as fermentation progresses, the environmental pH changes at different rates. At an initial pH above 7, the environment is more alkaline. However, as fermentation progresses, microorganisms metabolize nutrients present in the medium, leading to the production of

target products and other organic acids as byproducts. This leads to the pH of the medium decreasing toward a more neutral level [77]. It was observed that the prolongation of fermentation causes all the pH levels to decrease and converge at a near-neutral pH of 7.2–7.9. The trend observed in our study is consistent with findings in similar research. In a study examining the influence of the initial pH on hydrogen fermentation of food waste [59], it was observed that the initial fermentation pH, within the range of 5.0–9.0, spontaneously adjusted to approximately 6.0 over time without any pH control. Moreover, pH control was ascribed to protein degradation, a process believed to occur over an extended period when ammonia production exceeded acid production, as indicated by [78]. In a similar study focusing on the assessment of pH control during anaerobic co-fermentation of municipal waste, maintaining a neutral pH resulted in the consistent production of desired end products, including lactic acid, acetate, butyrate, and methane [79]. Likewise, another study on the effect of pH on the analysis of acidogenic properties of carbohydrate-rich wasted potato and microbial community showed that sustaining a neutral fermentation pH reduced the toxicity of undissociated volatile fatty acids, consequently, alleviating microbial inhibition [80].

Another plausible reason for the observed drop and stability in pH may either be because of the dissolution of CO_2 in the growth medium, cell death resulting from stress, nutrient depletion, or adverse conditions experienced by microbial cells [81]. During cell death, metabolic activities responsible for generating acids are halted, causing the pH to remain stable. Stability in the medium pH indicates that the alkalinity of the fermentation media provided a robust buffering capacity, effectively resisting fluctuations in pH throughout the fermentation process. As shown in Fig. 2, the final pH of the medium after 168 h remained within the neutral range. This maintenance of a neutral pH in the fermentation media is crucial for optimal microbial growth, as most microorganisms tend to thrive well in environments close to neutral pH [62].

3.2 Effect of pH on Ammonia Production over Time

Product yield during fermentation is influenced by several factors, including the pH of the fermentation medium and the duration of fermentation [82]. Both fermentation time [35] and pH [83] during microbial fermentation have been shown to affect ammonia yield. The importance of pH lies in its connection to the optimal activity of proteolytic hyper-ammonia-producing bacteria (HAB) [40].

The initial pH of the fermentation mixture can potentially influence the by-product generation during the fermentation process [59]. This suggests that ammonia production might be influenced by the initial pH of the reaction environment. Given that ammonia is a weak and volatile base [84], it undergoes partial dissociation, leading to the formation of both ammonium ions (NH_4^+) and hydroxide ions (OH^-) when introduced into a solution. Notably, under acidic conditions (low pH), the concentration of hydrogen ions (H^+) is elevated, prompting the equilibrium to shift towards the increased formation of ammonium ions (NH_4^+) from ammonia. Conversely, under alkaline conditions (higher pH), the concentration of hydroxide ions (OH^-) becomes more pronounced, driving the equilibrium towards favoring the generation of additional ammonia from ammonium ions [85]. Thus, the production of ammonia induces pH alterations, which may impact the growth and metabolic activity of the HAB. This dynamic between ammonia production and pH characterizes a major aspect of the fermentation process. Therefore, it adds up that pH variations could be used to induce microbial metabolism toward the production of targeted products, such as ammonia [67,86].

The effect of pH on ammonia yield over time was investigated by conducting a series of fermentation experiments under differing pH conditions (7–11) and periods (12–120 h) (Fig. 3).

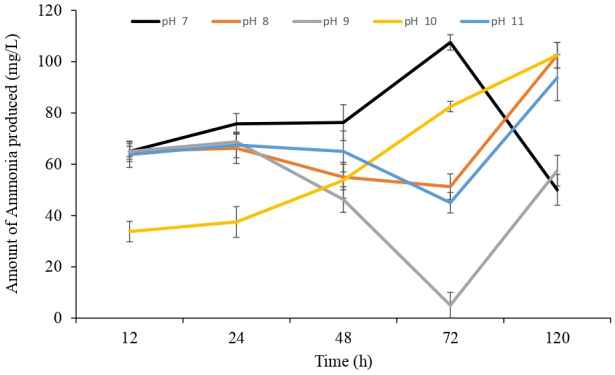

Fig. 3. Ammonia yield and pH levels during soy protein fermentation over time.

The result of ammonia yield during soy meal protein fermentation at various time intervals and pH levels is presented in Fig. 3. The results indicate that ammonia yield varies based on changes in pH and fermentation duration. pH levels 8, 9, and 11 displayed a fluctuating pattern over time, unlike pH 7 and 10, which were consistent. There was an initial increase in ammonia production within the first 24 h, followed by a gradual reduction between 24 and 48 h and a significant drop beyond 48 h. This notable decrease, observed at 72 h for pH 9 and 11 can be attributed to ammonia assimilation, a common characteristic of ammonia-producing bacteria [31,35,87,88]. However, ammonia production resumed beyond 72 h. This trend of delayed ammonia production is not practical for industrial purposes due to the potential economic losses associated with biological contamination from extended fermentation [89]. As such, pH levels 8, 9, and 11 are not considered feasible. The ob-

servation is consistent with our results (Fig. 1), where HAB proliferation favored pH concentrations of 7 and 10. Interestingly, even when the fermentation time increased beyond 72 h, ammonia production did not experience a major drop. Alternatively, pH concentrations of 7 and 10 demonstrated favorable ammonia production profiles. Both pH levels exhibited continuous ammonia production throughout fermentation, with high ammonia levels still evident at pH 10 after 120 h. This direct relationship between ammonia production and pH is in line with earlier studies where an increase in the pH *in vitro* also caused an increase in the rate of ammonia production [67]. Furthermore, certain phylum, such as actinobacteria in the rumen of ruminant animals, which show a positive relationship with pH may be present in the HAB consortium that is used during fermentation [90].

A neutral pH recorded the highest ammonia concentration (107.5 mg/L) at 72 h. However, after 72 h at pH 7, there was a noticeable decline in ammonia production due to nutrient depletion. Additionally, the accumulation of toxic metabolites could adversely affect the activity of the ammonia-producing bacteria, thereby reducing their output. Therefore, a fermentation duration of 72 h at a neutral pH is ideal for maximizing ammonia production during soy meal protein fermentation.

3.3 Effect of Varying Substrate Concentrations on Biological Ammonia Production

Substrate concentration is one of the major factors that impact fermentation outcome [91,92]. The effect of substrate concentration on product formation during microbial fermentation depends on many factors, including bacteria sensitivity to environmental conditions, fermentation duration, and substrate inhibition [92–96]. Specifically, the rate of biological ammonia production during HAB fermentation is linked to the nature of the substrate employed [47]. In a study to identify the most suitable substrate for biological ammonia production by HAB, pure HAB cultures were cultivated *in vitro* using five distinct substrates and their various combinations [31,51]. The substrates of soy protein isolate (SP), blood meal (BM), feather meal (FM), dried fish (DF), and yeast extract (YE), which are rich in organic nitrogen sources, were explored to discern the best protein substrate. It was observed that identical quantities of biological ammonia were generated from several combinations, including BM alone, BM and YE, SP and YE, FM and YE, DF alone, DF and YE, and YE alone. However, the highest biological ammonia concentration (7.23 mM) was achieved when soy protein isolates served as the substrate alone [51]. In the present study, soy meal protein isolate served as the primary substrate. However, to optimize fermentation performance and identify the ideal substrate concentration range, a thorough investigation is necessary [91].

To determine the most effective substrate level for enhancing biological ammonia production during HAB fermentation, we subjected varying concentrations of SMPI (2.5, 5, 10, 15, and 20%) to a fermentation process for 72 h. This research represents a pioneering investigation into the influence of different SMPI concentrations on biological ammonia production. The results, as shown in Fig. 4, suggested a clear and direct relationship between substrate concentration and biological ammonia concentration.

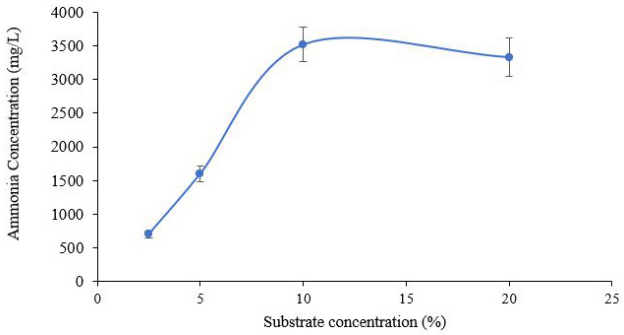

Fig. 4. Effect of substrate concentration on biological ammonia production at 72 h.

As the substrate concentration increased from 2.5% to 10%, so did the ammonia output, reaching its peak at ~3500 mg/L, with a substrate concentration of 10%. However, as the substrate concentration continued to rise beyond this point, a gradual decline in ammonia production became evident. This trend corroborates findings in similar studies examining the impact of substrate concentration on hydrogen production [94]. Increased sucrose (substrate) dosage that led to reduced H_2 yield was attributed to substrate inhibition [97,98]. Similarly, affirmed this pattern in their investigations on biohydrogen production and enhancement of hydrogen production from glucose, respectively [97,99]. Moreover, the accumulation of toxic byproducts during fermentation could have exerted inhibitory effects on product formation, further contributing to the observed decrease in ammonia concentration.

3.4 Effect of Inoculum Concentration on Biological Ammonia Production

Inoculum concentration, also known as cell size, is a principal factor that affects the production of bioactive compounds during fermentation [1,100]. Specifically, the rate and concentration of ammonia produced during HAB fermentation are affected by the size of the inoculum [1,47].

Bacteria possess an inherent robust morphological structure that prepares them for adapting to new environments [98]. As they enter a different environment, they utilize available nutrients to synthesize new enzymes, while curtailing the production of certain other enzymes [98,101–103]. Notably, research highlights that the initial size

of the microbes introduced as an inoculum into the fermentation medium influences the pace of genetic transformation, molecular composition, and microbial growth [98,104–108]. In fact, investigations have also shown that inoculum concentrations affect the lag time and the ability of the microbial population to initiate growth [98,109]. Occasionally, the lag phase might also produce a pseudo-lag phase, which is characterized by limited and non-viable inoculum concentrations [68,110]. In a study focused on estimating bacteria growth parameters through detection times, it was determined that as the inoculum cell count decreases, the lag time increases, particularly for inoculum levels ranging between 10^2 and 10^3 cells [111].

Limited research exists regarding the impact of the HAB inoculum concentration on soy meal protein fermentation for biological ammonia production, while the potential influence of increasing the inoculum concentration on enhancing biological ammonia production is unknown. Addressing this gap necessitates thorough investigations to explore the relationship between inoculum concentration and its effect on ammonia production during soy protein fermentation. Varying inoculum concentrations of 1, 2.5, 5, and 10% were inoculated into the media and monitored over different fermentation periods (0, 24, 48, 72, 120, and 168 h). Fig. 5 shows the effect of inoculum concentration on biological ammonia production over time.

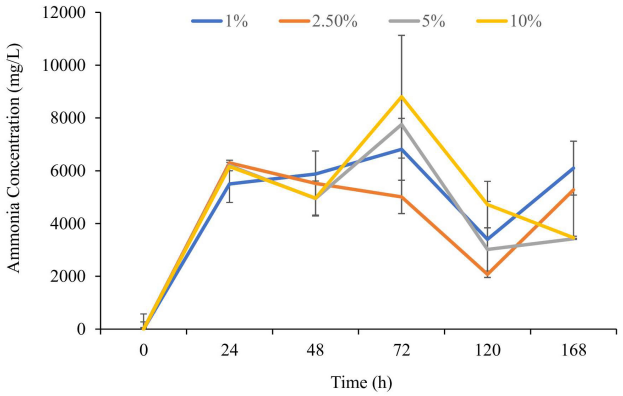

Fig. 5. Effect of inoculum concentration and time on biological ammonia production.

A zig-zag pattern was observed in the biological ammonia production profile. During the initial 24 h, a direct relationship was observed between the size of the inoculum and ammonia production, yet as the inoculum concentration grew, so did the ammonia output. The ammonia production reduced post-24 h before gradually increasing after 48 h. It was observed that, except for the 2.5% inoculum concentration, all inoculum concentrations reached their peak performance at 72 h. However, ammonia production began to decline after 72 h. Several factors could be responsible for this decline. As the fermentation process progresses, essential nutrients in the medium might get exhausted, leading to reduced microbial activity and, consequently, decreased ammonia production. It is also possible that the accumulation of products such as ammonia and other metabolic byproducts has reached levels that are inhibitory to the HAB. Microbial cells might also enter a stationary or death phase characterized by the lysis of cells, which leads to the release of intracellular enzymes that might break down ammonia or alter its production rate. Another probable reason for the observed decline is ammonia assimilation by the microbes [31,35,87,88].

The highest biological ammonia concentration, ~8805 mg/L, was achieved using a 10% inoculum concentration and a fermentation time of 72 h. Remarkably, this result aligns with the suggested 5–10% fermentation inoculum concentration range [68]. In a study on the effect of inoculum concentration on cell growth, acid production, and curd formation during milk fermentation by *Lactobacillus plantarum Dad 13*, it was noted that an inoculum concentration increases from 1 to 5% enhanced the formation of products [112].

Likewise, an optimum inoculum concentration of 10% was observed in a study on the effect of inoculum concentration on solid-state fermentation of pearl millet (*Pennisetum glaucum*) by *Rhizopus oligosporus.*

It was observed that a 1% inoculum concentration yielded more biological ammonia compared to a size of 2.5%. This is not unusual since high microbial populations can produce inhibitory compounds or metabolic byproducts that hinder product synthesis [113]. Further, smaller inoculum concentrations may produce fewer inhibitory substances, thereby creating a more favorable environment for product formation.

Overall, our results suggest that there is a proportional relationship between inoculum concentration and the amount of ammonia produced during fermentation. Therefore, optimizing inoculum concentration is a crucial factor for maximizing biological ammonia production. Based on this result, an inoculum concentration and fermentation time of 10% and 72 h, respectively, would be selected for further fermentation studies.

4. Conclusions

The present study is a pioneering effort to assess the impact of key factors—pH, alkalinity, substrate concentration, and inoculum concentration—on the anaerobic fermentation of soy meal protein for biological ammonia production. Notably, the alkalinity of the fermentation medium emerged as a vital factor, effectively buffering against pH fluctuations. The results emphasized the significance of maintaining a neutral pH of 7, a substrate concentration of 10%, and an inoculum concentration of 10% to achieve optimal HAB proliferation and, consequently, maximize biological ammonia production (~8800 mg/L). These results provide invaluable insights into the anaerobic biological

production of ammonia via HAB fermentation using soy meal protein as the substrate. Building upon these findings, future research will investigate further process optimization aimed at harnessing the full potential of biological ammonia production for industrial applications. Additionally, a wider range of processing factors are worth further investigation. This research sets a promising foundation for sustainable and economically viable ammonia production, aligning with the recent growing demand for circular economy-based solutions.

Availability of Data and Materials

All data points generated or analyzed during this study are included in this article and there are no further underlying data necessary to reproduce the results.

Author Contributions

IB, investigation, experimental, data curation, formal analysis and writing original draft; AA, formal analysis, data curation and writing original draft; TM, formal analysis, data curation and writing original draft; EM, project administration, methodology, validation; AH, conceptualization, funding acquisition, project administration, methodology, supervision, and validation. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work, ensuring its accuracy and integrity. All authors read and approved the final manuscript. All authors contributed to editorial changes in the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Authors would like to acknowledge the North Dakota Soybean Council for their financial aid. Gratitude is also extended to the North Dakota State University, Department of Agricultural and Biosystems engineering for allowing the use of its laboratory facilities and equipment.

Funding

This research was funded by North Dakota Soybean Council and State Board of Agricultural Research and Education with grant numbers FARG0034901 and FARG090432, respectively.

Conflict of Interest

The authors declare no conflict of interest.

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