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Overproduction of ammonia by a local isolate of *Staphylococcus aureus* in a cheaply fabricated medium

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ABSTRACT: Upon screening a number of formulated media for ammonia overproduction by *Staphylococcus aureus*, an ammonia concentration of 13.09 mg/ml was obtained. the medium used was 10% concentration of fish hydrolysate prepared by 5% HCl digestion. Ammonia yield on synthetic peptone broth was 12.75 mg/ml. Physical parameters such as incubation time, temperature, effect of aeration and agitation were investigated for an optimization of the bioprocess.

Key Words: Ammonia; Overproduction; Metabolites; Formulated peptone; *Staphylococcus aureus*.

Introduction

Ammonia production is the first step in the manufacture of nitrogen fertilizers (1) the importance of which is critical for crop growth and yield. The industrial production is based on Haber-Bosch process. A mixture of nitrogen and hydrogen is subjected at a temperature of 500^{o}C and at high pressures in the presence of a catalyst (2). Some species of the rumen bacteria are known to be proteolytic (3) and possess the enzymatic ability to break down peptides and amino acids to produce ammonia. *Clostridium aminophilus, Peptostreptococcus anaerobicus* and *Clostridium sticklandii* have been implicated in hyperproduction of ammonia (4).

Overproduction of metabolites through the conventional recombinant DNA technology has some limitations. The maintenance and expression of several copies of a gene within a host requires certain amount of the cell resources that impair the cell normal metabolic functioning. This is called a metabolic burden or a metabolic drain (5). Metabolic burden increases with both the size and the copy number of the new gene (6), and consequently lowers the expression level of the gene (7). A second problem faced with this pathway of overproduction is the production lethality (8) in which the foreign proteins jam the export sites of the cell membrane and prevent proper localization of essential proteins.

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Another efficient approach used in metabolite overproduction lies in the fact that the composition of the culture medium affects the product formation by microorganisms. Microbial metabolic fluxes are highly dependent on growth environment and therefore can be manipulated by a careful choice of the culture conditions (9). This is generally carried out in various limited chemostat culture.

The objective of the work reported here is to overproduce ammonia using a low cost formulated medium by a local isolate of *Staphylococcus aureus*.

Materials and Methods

Isolation of microorganisms

A decaying corn root was suspended in nitrogen free broth containing 0.01% KH_2PO_4 for three days. A drop of the solution was streaked on solid medium containing 0.01 KH_2PO_4, 0.02% $MgSO_4$, agar powder and water. Pure colonies of several organisms were obtained by subculturing repeatedly on yeast extract agar. the organisms were screened for the ability to produce ammonia as follows: each organism was cultured in peptone water and incubated for 24h at 30°C. Gentle heat was applied to each culture and fume giving up allowed to react with a moist litmus paper. the isolate with the highest rate of ammonia production was characterized using routine biochemical test.

Assay for ammonia production

Tests for ammonia production were carried out on three different media of the following composition:

A1: 15% (w/v) peptone + 0.25% (w/v) yeast extract.
A2: 15% (w/v) peptone + 4% (w/v) glucose.
A3: 15% (w/v) peptone alone.

A 24h old culture of the organism on yeast extract agar plates were washed into 24 ml each of the peptone broth A1, A2 and A3. Cultures were incubated at 30°C for 24h. A moist litmus paper was used to test the ammonia production in each sample.

Estimation of ammonia concentration in cultures

The method of Basset et al. (10) was adopted to quantify the ammonia in the supernatant and is based on extrapolation of ammonia concentration from spectrophotometric readings of the culture supernatant on a standard curve. To obtain the standard curve, the absorbance readings of six samples of different concentrations of NH_4Cl were measured using Spectronic 2oD at 410 nm, the wavelength for the maximum absorbance of ammonia earlier obtained. These readings were plotted against the ammonia concentrations and the resulting regression line was the standard curve used for estimation of ammonia from spectrophotometric readings in Fig. 1, Tables 3 – 5.

Optimum physical conditions for ammonia production

The optimum physical conditions for *S. aureus* growth with ammonia production were determined. The incubation time for maximum production of ammonia was evaluated using seven samples of peptone broth inoculated with bacterial suspensions obtained from 24h old plates. these were incubated at 30°C. Samples were centrifuged at 24h interval for a period of 168h of incubation at 2,500 rpm for 15 min. The quantification of the ammonia in the supernatant was done using the method of Basset et al. (10). To determine the optimum temperature for growth and ammonia production, three samples of 25 ml each of peptone broth were inoculated with bacterial suspensions obtained from 24h old plates. these were incubated at 25°C, 30°C and 35°C. Fifteen ml of each sample was centrifuged and the ammonia content of the supernatant quantified using the method of Basset et al. (10). The effect of aeration and agitation on ammonia production was investigated in 100 ml peptone broth inoculated with bacterial suspension washed off from 24h old plate. Filtered air was supplied using an air pump which also served as the source of

agitation. A control culture was set up without aeration. Both cultures were incubated at room temperature for 24h. Five ml of each culture was centrifuged at 2,500 rpm for 15 min and the ammonia content of the supernatant estimated using the method of Basset et al. (10).

Evaluation of locally formulated peptone media for ammonia production

Three locally available substrates were initially considered for use in peptone preparation. These were fish, meat and soybean. The meat and the fish substrates were separately chopped into bits. The soybean was grounded into fine powder. Acidic digestion of the substrates was done at 5% and 10% HCl concentrations. The following experimental media were set up:

M1: 80g of minced meat + 100 ml of 5% HCl.
M2: 80g of minced meat + 100 ml of 10% HCl.
M3: 80g of minced fish + 100 ml of 5% HCl.
M4: 80g of minced fish + 100 ml of 10% HCl.
M5: 80g of powdered soybean + 100 ml of 5% HCl.
M6: 80g of powdered soybean + 100 ml of 10% HCl.

These media were autoclaved at 121^{o}C for 120 min. This was followed by filtration to remove the undigested particles. The pH was raised to 7.0. Peptone from soybean substrate was later discarded based on its high content of carbohydrate, being a plant peptone. each of the hydrolysates obtained was diluted to obtain three concentrations, viz. 10%, 50% and the stock. Fr sterilization, the hydrolysates were dispensed at 25 ml into bottles. Inoculation was done by washing a 24h old plate of the *S. aureus* into each of the media. The cultures were incubated for 24h at 30^{o}C, the optimum temperature for ammonia production. Cultures were centrifuged at 2,500 rpm and the ammonia concentration of the supernatant was determined by the method of Basset et al. (10).

Results and Discussion

Identification of the isolate

The results of identification of the isolate with the highest level of ammonia production show that it is Gram-positive, spherical in shape and non motile. The organism is oxidase negative and produces coagulase (Table 1). It hydrolyses starch and gelatin and ferments glucose, lactose, fructose and xylose. From all these, it was confirmed to be a strain of *Staphylococcus aureus*.

Tests for ammonia production

The tests for ammonia production which were carried out on synthetic peptone media A1, A2 and A3 showed that the organism could not produce ammonia in peptone broth containing glucose (Table 2). An explanation to this may be derived from the understanding of ammonia production pathways.

The enzymatic pathway of ammonia production begins with the activity of proteolytic enzyme (11) and it is an effect of deamination of amino acids giving off ammonia and free carbon radicals. The resulting ammonia is normally used for structure development. If a source of carbohydrate is absent in the medium, the organism will utilize the carbon residues for energy, since more carbon is required than nitrogen for structure. In so doing, ammonia will accumulate in the medium. In the presence of a fermentable carbohydrate, the organism utilizes the ammonia for structure and this does not accumulate in the medium (12). Based on this, the use of soybean peptone for ammonia production was dropped. According to Bridson and Brecker (13) the vegetable based peptones contain high rate of carbohydrate.

Physical condition for optimum ammonia production

The incubation time for optimum production of ammonia was 24h after which production began to decline (Fig. 1). The optimum temperature for *S. aureus* growth and ammonia production was obtained at

30°C (Table 3). In aerated culture, higher rate of ammonia production was obtained than in the non aerated one (Table 3). The agitation effect from aeration homogenized the culture and prevented a local depletion of some key nutrients or a local build up of the lethal values of some physical parameters like the pH.

Table 1: Biochemical analysis of the hyper-ammonia producing isolate.

Catalase	+
Coagulase	+
Oxidase	–
Citrate utilization	–
Pigmentation	+
H_2S production	+
Starch hydrolysis	+
Gelatin hydrolysis	+
MR	–
VP	–
Indole production	–
Glucose	A
Lactose	A
Sucrose	A
Fructose	A
Maltose	A
Dextrin	A
Xylose	A
Ducitol	A

A = acid production; + = positive; – = negative.

Table 2: Ammonia production by *S. aureus* on synthetic peptone.

Medium code	Medium composition	NH_3
A1	Peptone + yeast extract	+
A2	Peptone + glucose	–
A3	Peptone alone	+

+ = Presence of ammonia; – = Absence of ammonia.

Ammonia production in fabricated media

In evaluating the fabricated peptone media for ammonia production by *S. aureus*, some formulations produced higher rate that the synthetic peptone medium. For instance, with 10% hydrolysate of fish substrate prepared with 5% HCl, an ammonia concentration of 13.09 mg/ml was obtained (Table 4) while the synthetic peptone broth culture gave 12.75% mg/ml of ammonia. With the meat substrate, the highest ammonia concentration (12.94 mg/ml) was obtained when 50% hydrolysate from 5% HCl digestion was used. When fish and meat hydrolysates obtained with 5% HCl digestion were mixed at the rate of 1:1, the highest ammonia concentration (12.83 mg/ml) was obtained (Table 4).

Table 3: Effects of aeration and temperature on ammonia production by *S. aureus*.

	Media	Absorbance at 0h	Absorbance at harvesting time	Ammonia concentration (mg/ml)
Aeration time	E1	0.11	0.19	12.77
	E2	0.11	0.14	12.20
Temperature	C1	0.038	0.03	11.80
	C2	0.038	0.09	12.46
	C3	0.038	0.05	12.02

Each value is the average of two readings.

Table 4: Ammonia production by *S. aureus* when different concentrations of hydrolysates prepared with 5% HCl digestion were used.

Substrate hydrolysate concentration (%)	Difference in ammonia absorbance (between 0h and 24h) at 410 nm	Ammonia concentration (mg/ml)
Meat		
10	0.069	12.65
50	0.095	12.94
100	0.027	12.19
Fish		
10	0.108	13.09
50	– ve	–
100	– ve	–
Mixture (Fish/Meat 50:50)		
10	0.060	12.55
50	0.085	12.83
100	0.052	12.47

Each value is the average of two readings.

There is a preferential ability of the organism for high ammonia production with 10% concentration of fish hydrolysate prepared by 5% HCl digestion. The fish substrate contained trace amounts of glycogen. Considering the energetics of microbial growth and product formation, Tempest and Nejissel (14) hypothesized that there are three types of energy consuming reactions: The net synthesis of new materials, the maintenance of the cell integrity and the energy spilling reactions. Energy spilling reactions occur mainly when the cell is in a state of energy surplus. It is almost unlikely that *S. aureus* might have embarked on energy spilling reactions while being in relative state of energy shortage. In the light of this, it

can be argued that ammonia production by *S. aureus* in fish peptone was preceded by a significant build up of biomass.

The hydrolysates obtained with 10% HCl digestion have a low ammonia yield (Table 5). This sensitivity of *S. aureus* to the peptone digestion's level may be due to the distribution of the total nitrogen in the peptone. According to Bridson and Brecker (13), the digestion level characterizes the distribution if nitrogen into primary proteose nitrogen, free amino acid nitrogen, secondary proteose nitrogen and ammonia nitrogen. These authors further stated that when identical organisms are grown on a solid media containing peptone which differs in these distributions, they exhibit a variation in their metabolism. In some cases, the supply of peptides in a certain order of amino acid combination may be critical in product formation.

The potentialities of improving the biological production of ammonia by manipulation of microbial metabolic fluxes through medium formulation remain immense. Hence a need to consider this approach as an alternative to curb the growing cost of production of nitrogen fertilizers.

Table 5: Ammonia production by *S. aureus* when different concentrations of hydrolysates prepared with 10% HCl digestion were used.

Substrate hydrolysate concentration (%)	Difference in ammonia absorbance (between 0h and 24h) at 410 nm	Ammonia concentration (mg/ml)
Meat		
10	0.074	12.75
50	– ve	–
100	– ve	–
Fish		
10	– ve	–
50	– ve	–
100	0.025	12.17

Each value is the average of two readings.

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