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EFFECT OF AMMONIUM SULPHATE ON THE SPORULATION OF *BACILLUS THURINGIENSIS* SUBSP. *AIZAWAI* SN2 (A LOCAL ISOLATE) DURING BATCH FERMENTATION

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Abstract. The effect of ammonium sulphate (as the sole inorganic nitrogen source) on the sporulation of *Bacillus thuringiensis* subsp. *aizawai* strain SN2 was investigated in batch fermentation. The spore percentage of 76 % was achieved at 96 h after inoculation into a medium containing initial cencentration of 1.5 gL^{-1} ammonium sulphate. In another experiment, the maximum spore percentage of 82 % was obtained after 96 h inoculation in a medium with initial concentration of 1.5 gL^{-1} $(NH_4)_2SO_4$ followed by an addition of 3.0 gL^{-1} $(NH_4)_2SO_4$ after 6 h of fermentation. The increase in *Bacillus thuringiensis* spore percentage was not a function of total nitrogen content in the medium but was a function of the time nitrogen being added.

Keywords: *Bacillus thuringiensis* subsp. *aizawai* strain SN2, timing of nutrient addition, batch fermentation

Abstrak. Kesan penambahan ammonium sulfat, $(NH_4)_2SO$, (sebagai sumber nitrogen bukan organik tunggal) terhadap pensporaan *Bacillus thuringiensis* subsp. *aizawai* strain SN2 dalam kultur kelompok telah dikaji. Peratus spora tertinggi (76 %) dikesan selepas 96 j pengkulturan dalam medium yang mengandungi 1.5 gL^{-1} $(NH_4)_2SO_4$. Peratus spora tertinggi sebanyak 82 % juga dikesan dalam sampel 96 j apabila medium yang mengandungi 1.5 gL^{-1} $(NH_4)_2SO_4$ ditambah dengan 3.0 gL^{-1} $(NH_4)_2SO_4$ pada jam keenam selepas mula fermentasi. Peningkatan peratus spora didapati tidak berkaitan dengan jumlah kandungan nitrogen tetapi berkaitan dengan masa penambahan sumber nitrogen.

Kata kunci: *Bacillus thuringiensis* subsp. *aizawai* strain SN2, masa penambahan nutrien, kultur kelompok

1.0 INTRODUCTION

Bacillus thuringiensis (Bt) bioinsecticide was reported as one of the most successful biotechnology product that are available in the market [1] due to it's potential as specific pest control in the agricultural sector. Therefore, studies to determine the medium composition for enhanced Bt production become important. Thus, media consisting of carbon, nitrogen, phosphorus, and potassium sources need to be constantly improved [2]. Studies on the use of substrate such as corn-steep liquor or peptones [3],

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broiler litter extracts [1] and gruel-based media [4] to produce Bt bioinsecticide have been reported. However, all the reports focused on the usage of crude organic nitrogen sources in the hope of reducing cost of production. The effect of a sole inorganic nitrogen source to enhance Bt sporulation such as ammonium sulphate, a cheap and easily available inorganic nitrogen source has never been fully investigated although the existence of ammonium ion, NH_4^+ and to some extent sulphate ion, SO_4^{2-} in the medium was reported to stimulate sporulation [4]. The NH_4^+ ions are known as an easily utilizable source of inorganic nitrogen for bacterial growth compared to other source of nitrogen such as nitrate, NO^{3-} where the ability of microorganism to assimilate it is more restricted [6]. Medium containing ammonium sulphate was also reported to give higher biomass and higher (endotoxins production compared to other nitrogen sources such as peptones [5]. All reports stated that NH_4^+ was employed at the all reports employed NH_4^+ at the beginning of batch fermentation and no investigation was reported with respect to addition of NH_4^+ during fermentation.

In this study, we would like to report the effect of a timed addition of ammonium sulphate (as the sole inorganic nitrogen source) on the sporulation of *Bacillus thuringiensis* subsp. *aizawai* strain SN2 during batch fermentation to increase percentage of spore production.

2.0 MATERIALS AND METHODS

2.1 Microorganism

The microorganism employed was a locally isolated *Bacillus thuringiensis* subsp. *aizawai* SN2 obtained from the School of Bioscience and Biotechnology, Universiti Kebangsaan Malaysia. The bacterium was subcultured at 30°C on nutrient agar (SIGMA) for 48 h and stored at 4°C as stock culture.

2.2 Inoculum and Cultivation Medium

The cultivation medium consisted of (gL^{-1}) glucose, 5.00; yeast extract, 1.00; $(NH_4)_2SO_4$, 1.50; K_2HPO_4, 0.32; $MgSO_4.7H_2O$, 0.30; $CaCl_2.2H_2O$, 0.08; $MnSO_4.H_2O$, 0.05; $FeCl_3.6H_2O$, 0.001; $ZnSO_4.7H_2O$, 0.005; $CuCl_2.2H_2O$, 0.005; H_3BO_3, 0.00006; $NaMoO_4.2H_2O$, 0.00002; and distilled water. The final pH of the medium was adjusted to 7.0 – 7.2 using 1M NaOH (Modified from Sakharova, 1984) [8].

2.3 Inoculum Preparation

A loop full of *B. thuringiensis* was used to inoculate 100 mL medium in a 250 mL Erlenmeyer flask and was incubated on an orbital shaker at 150 rpm and 30°C for 14 hours. A 10 ml of spore suspension consisting 10^7 spore/ml was used as inoculum.

2.4 Batch Culture Fermentation

Fermentation was conducted in a 500 mL Erlenmeyer flasks each containing 200 mL medium. Different concentrations of $(NH_4)_2SO_4$ (1.5 gL^{-1}, 3.0 gL^{-1}, 4.5 gL^{-1}) were added into each flask which was then inoculated with 5 % (v/v) of spore suspension (10 mL). The spore suspension was pre-heated at 80°C for 10 minutes prior to inoculation. All flasks were incubated for five days on an orbital shaker at 150 rpm and 30°C. Duplicate flasks were set up for each experiment.

The experiment was divided into two sets. In the first set, different concentrations of $(NH_4)_2SO_4$ was added only at the beginning of the experiment. Sample was taken at 48 h, 72 h, and 96 h of cultivation to estimate for total viable cell, spore counts and the spore percentage.

In the second set, 1.5 gL^{-1} $(NH_4)_2SO_4$ was added at the beginning of the experiment and 1.5 gL^{-1} or 3.0 gL^{-1} $(NH_4)_2SO_4$ after 6 h was added of fermentation. Only samples at 72 h and 96 h of cultivation were taken for the estimation of total viable cell counts, spore and spore percentage. Samples at 0 h, 3 h, 8 h, 72 h, and 96 h were taken for estimation of the total nitrogen content.

2.5 Analytical Procedures for Estimation of TVCC and SC

Samples of diluted spore suspension were grown on nutrient agar following the pour plate method. The corresponding dilutions of spore suspension was heated at 80°C for 10 minutes for the determination of heat resistant spores. Duplicate dilution was set up for each of the experimental.

Growth was determined by the measurements of optical density (OD) using a "Hitachi U1100 Spectrophotometer" at the wavelength of 550nm for every 2 hours up to 10 hours and followed by every 24 hours until the end of the experiment. Result from optical density (O.D) measurements was used to determine the beginning of the stationary phase of growth, specific growth rate, (μ) and doubling time, (t_d). Culture doubling times were determined from the slope of plot In OD against fermentation time [7]. The relationship between td and u is summarized as follow:

$$\text{Doubling time, } (t_d) = \ln 2 / \mu \quad (1)$$

Samples for total nitrogen content were sent to the Food Quality Research Unit, UNIPEQ (Universiti Kebangsaan Malaysia) for the analysis via kjeldahl method.

3.0 RESULTS AND DISCUSSION

3.1 Effect of the Timed-addition of Ammonium Sulphate [$(NH_4)_2SO_4$] on Sporulation of *B. thuringiensis* subsp. *aizawai* SN2

Figures 1, 2, and 3 showed the effects of different concentrations of $(NH_4)_2SO_4$ added at the beginning of the experiment (t = 0 h) on total viable cell counts (TVCC), spores counts (SC), and spore percentage at 48 h, 72 h, and 96 h of incubation. Medium containing 1.5 gL^{-1} $(NH_4)_2SO_4$ showed the highest values of TVCC (Figure 1) and SC (Figure 2) compared with other concentrations tested. The highest value of TVCC and SC detected were 2.85×10^7 CFU mL^{-1} and 0.92×10^7 spore mL^{-1} at 48 h of cultivation respectively whilst the highest spore percentage (76 %) was recorded at 96 h. The addition of $(NH_4)_2SO_4$ at concentration greater than 1.5 gL^{-1} was found to decrease the spore percentage as well as TVCC and SC.

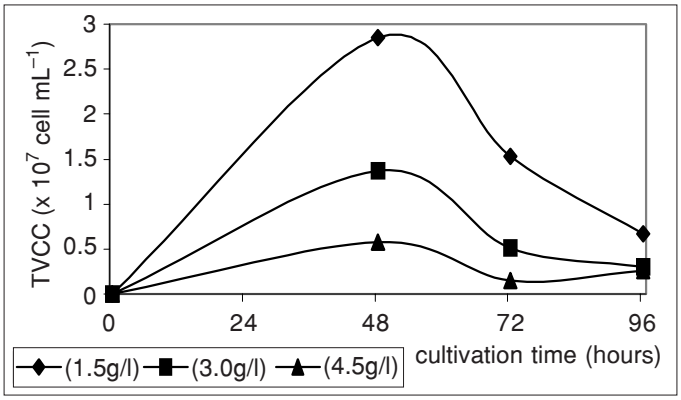

Figure 1 The effects of various concentrations of $(NH_4)_2SO_4$ added at the beginning of experiment (t = 0 h) on total viable cell counts (TVCC)

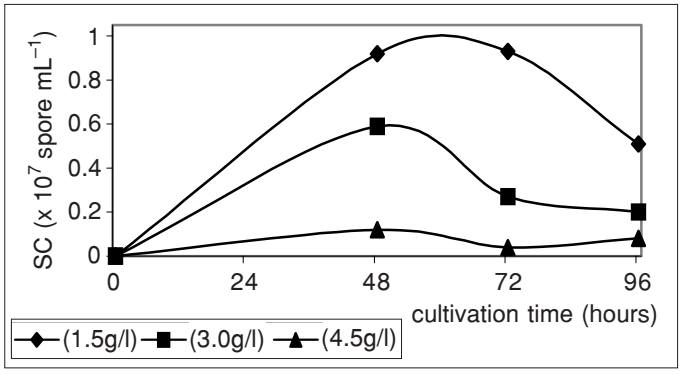

Figure 2 The effects of various concentrations of $(NH_4)_2SO_4$ added at the beginning of experiment on spores counts (SC)

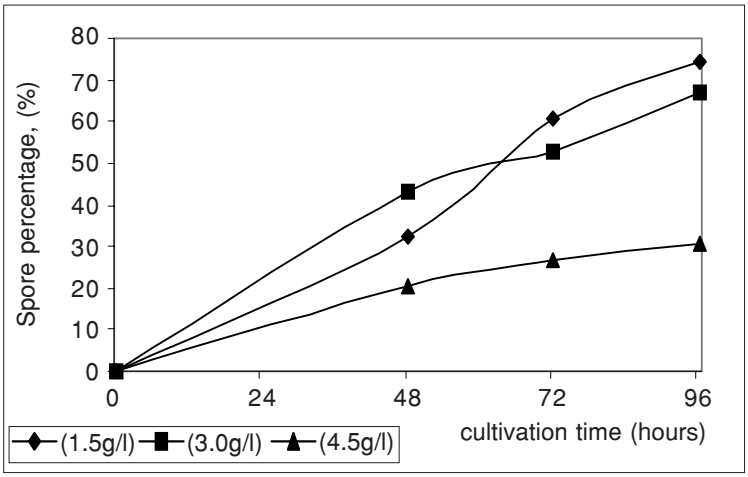

Figure 3 The effects of various concentrations of $(NH_4)_2SO_4$ added at the beginning of experiment on B .thuringiensis spore percentage

Figure 4, 5, and 6 showed the effects of an addition of 1.5 gL^{-1} and 3.0 gL^{-1} of $(NH_4)_2SO_4$ after 6 hours of fermentation on TVCC, SC and spore percentage respectively.

The highest spore percentage (82 %) was recorded at 96 h of cultivation using medium containing 1.5 gL^{-1} $(NH_4)_2SO_4$ added at the beginning of fermentation followed by an addition of 3.0 gL^{-1} $(NH_4)_2SO_4$ after 6 h fermentation (Figure 5). Results showed that the addition of $(NH_4)_2SO_4$ have the potential to increase the spore percentage. The results obtained were in agreement with Hardwick and Foster

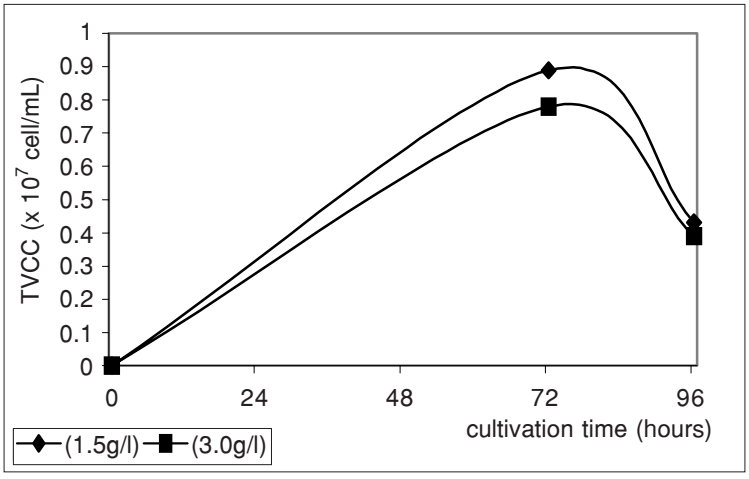

Figure 4 The effects of the addition of 1.5 gL^{-1} $(NH_4)_2SO_4$ at the beginning of experiment followed by an addition of 1.5 gL^{-1} or 3.0 gL^{-1} $(NH_4)_2SO_4$ after 6 h of cultivation on TVCC

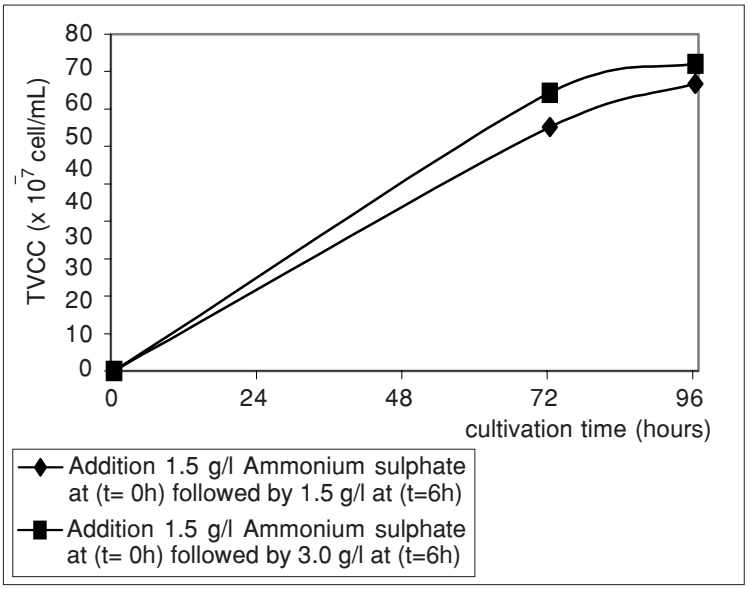

Figure 5 The effects of the addition of 1.5 gL^{-1} $(NH_4)_2SO4$ at the beginning of experiment followed by an addition of 1.5 gL^{-1} and 3.0 gL^{-1} $(NH_4)_2SO_4$ after 6 h of cultivation on spore percentage

(1952) [3] They had demonstrated that the cells of *B. mycoides* obtained from a medium containing low nitrogenous material failed to sporulate when shaken in distilled water but they were readily sporulated in a nitrogen-rich medium. They concluded that a cell impoverished with protein content would lose its ability to sporulate and this could be considered as an additional evidence that spore proteins were synthesized from cellular nitrogenous materials.

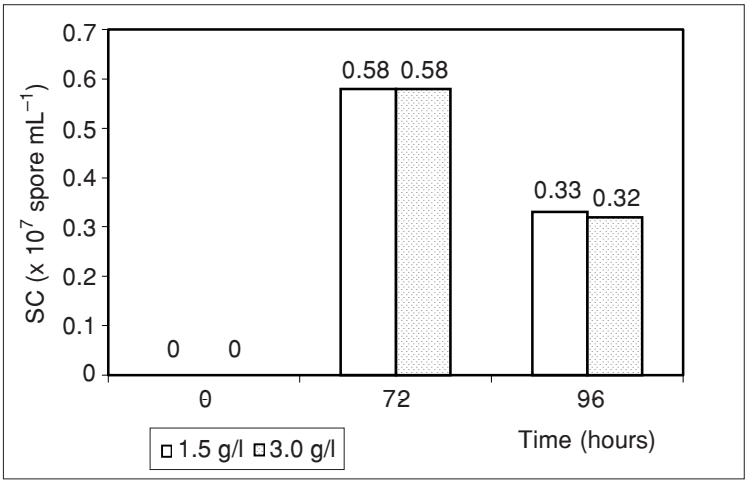

Figure 6 The addition of 1.5 gL^{-1} $(NH_4)_2SO_4$ at the beginning of the experiment followed by an addition of 1.5 gL^{-1} and 3.0 gL^{-1} $(NH_4)_2SO_4$ after 6 h of cultivation on *B. thuringiensis* spore count.

Table 1 Specific growth rate, (μ) and doubling time, (t_d) in the batch culture with addition of different concentrations of $(NH_4)_2SO_4$ at the beginning of experiment

$(NH_4)_2SO_4$ concentration at the begining of the experiment, (t = 0 h)	Specific growth rate (μ), hour^{-1}	Doubling time (t_d), minute
1.5	0.31	132
3.0	0.36	116
4.5	0.27	152

The addition of 3.0 gL^{-1} $(NH_4)_2SO_4$ gave the best value for the specific growth rate, ((0.36h^{-1}) and doubling time, t_d (116 minute) compared to other concentrations tested (Table 1). The addition of 4.5 gL^{-1} $(NH_4)_2SO_4$ was fund inhibiting, as indicated by its lowest value of specific growth rate, μ (0.27h^{-1}) and longest doubling time, t_d (152 minute) compared to those in 1.5g/L and 3.0g/L $(NH_4)_2SO_4$. Therefore, the concentration of 3.0 gL^{-1} $(NH_4)_2SO_4$ represented the maximum addition of inorganic nitrogen source before catabolic repression sets in.

3.2 Effect of Total Nitrogen Content on Sporulation

Table 2 showed the effect of medium containing 1.5 gL^{-1} $(NH_4)_2SO_4$ with an addition of 1.5 gL^{-1} and 3.0 gL^{-1} $(NH_4)_2SO_4$ after 6 h fermentation on the percentage of total nitrogen content and spore percentage at 96 h batch culture fermentation. The difference in total nitrogen content throughout the experiment was not significant (P = 1.000). Nevertheless, spore percentage increased to 74 % from 65 % after 72 h fermentation as a result to the addition of 3.0 gL^{-1} $(NH_4)_2SO_4$. This could mean that the nitrogen

Table 2 Addition of 1.5 gL^{-1} $(NH_4)_2SO_4$ at the beginning of the experiment followed by addition of 1.5 gL^{-1} (A) and 3.0 gL^{-1} (B) $(NH_4)_2SO_4$ after 6 h fermentation on the total nitrogen content and spore percentage in five days batch culture fermentation

Cultivation time (h)	Total nitrogen content (%)		Spore percentage (%)	
	A	B	A	B
0	0.07	0.07		
3	0.11 6 addition of 1.5 gL^{-1} $(NH_4)_2SO_4$ (20 ml)	0.11 addition of 3.0 gL^{-1} $(NH_4)_2SO_4$ (20 ml)		
8	0.13	0.13		
72	0.14	0.15	65	74
96	0.10	0.10	77	82

assimilated contributed to the extent of sporulation and may very well increase toxicity. The highest spore percentage of 82 % was obtained in experiment with the addition of 3.0 gL^{-1} $(NH_4)_2SO_4$.

4.0 CONCLUSIONS

Medium containing 1.5 gL^{-1} $(NH_4)_2SO_4$ at the beginning of the experiment produced a higher spore percentage (76 %) compared to those containing 3.0 gL^{-1} and 4.5 gL^{-1} $(NH_4)_2SO_4$. However, addition of 3.0 gL^{-1} $(NH_4)_2SO_4$ after 6 h fermentation result in the highest spore percentage of 82 % at 96 h of cultivation. Hence, the addition of inorganic nitrogen source during batch fermentation provided a simple means to increase toxicity of *B. thuringiensis* culture.

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