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Optimization of sporulation conditions of *Bacillus sublilis* as potential probiotics strain

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ABSTRACT

Probiotics are used as a substitute for antibiotic growth promoters in the chick industry. In the present study, conditions for the sporulation of *Bacillus subtilis*, a probiotic strain, are optimized. Overnight grown inoculum (1%) was used for the cultivation of *B. subtilis* in NB medium followed by 24-48 hours incubation in a shaking incubator at 30°C. Spore staining and microscopy were performed at different time intervals to find out the sporulation status and time required for the completion of sporulation. However, sporulation of the *Bacillus* strain completed after 22 hours and the total viable count (TVC) was found to be 8.3 x 10^{23} CFU/ml (OD 1.872). Similarly, optimization of temperature (30°C) and pH (7) was carried out that reduced the sporulation period up to 18 hours. The total viable count was 6.4 x 10^{12} CFU/ml. The acid tolerance of the *B. subtilis* was also determined at pH 2 and TVC after the acid treatment was 6.4 x 10^{12} CFU/ml. It depicts the ability of bacteria to withstand the acidic conditions.

Keywords: Probiotics, Optimization, Spores, *Bacillus subtilis*, Poultry, Growth enhancers

INTRODUCTION

The probiotics are living microbial feed supplements which help in the host animal by enhancing its intestinal stability. The word probiotic is obtained from the Greek word meaning, “For life” (Reid *et al.,* 2003). According to the recent definition, as suggested by the World Health Organization (WHO) and Food and Agriculture Organization (FAO), probiotics are the combinations of live microorganisms which when administered in an appropriate amount confer a health benefit on the host. Probiotics increase beneficial gut microflora and prevent the colonization of pathogenic bacteria in the gastrointestinal tract. Probiotics have many attributes like non-pathogenicity, ability to colonize and reproduce and also bear the acidity of bile and stomach. Today, these are commonly used in health-promoting “functional foods” for humans (Vijayaram & Kannan, 2018).

Probiotic administered in the feed helps in improving and stimulating the immune system by increasing intestinal microflora and their secretions help in maintaining the overall environment of the gastrointestinal tract (Adil & Magrav, 2012). Mechanism of pathogen inhibition may include stimulation of the immune system, competition for available nutrients, and direct antimicrobial effects by secretion of inhibitory substances or competition for adhesion receptors to intestinal epithelium (Lee *et al*., 2010).

The poultry industry plays a key role in the economies of many countries. However, this industry is continuously exposed to several threats in large-scale facilities including exposure to diseases, stressful conditions and deterioration of environmental conditions. These often lead to serious economic loss. Prevention and control of diseases, during recent decades, has led to a substantial increase in the use of veterinary medicines. Thus, alternative approaches are essential to maximizing the performance of broiler (Tactacan *et al.*, 2013). Probiotics are used as a substitute for antibiotic growth promoters in the chick industry.

Probiotics especially *B. subtilis*, when administered in the chick, helps in the maintenance of this mucin secretion and thus increase intestinal microflora but it also enhances various other function like weight gain, improve

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growth, helps in feed conversion, improve bird performance and improved intestinal function to properly digest food (Dalloul *et al*., 2003; Vila *et al*., 2009; Mountzoris *et al.*, 2010).

B. subtilis have the advantage of being used as a superior feed-additive for poultry and pigs due to their big genome size and spore-forming ability. These properties make them resistant and stable inside the bird's gastrointestinal tract and thus bacteria show better growth (Hmani *et al*., 2017).It has also been shown that *Bacillus* species can improve the sensory characteristics of broiler meat and promote meat quality (Park & Kim, 2014). The reduction in fat and cholesterol level and increased protein content in chicken meat supplemented with *Bacillus subtilis* has also been observed (Kral *et al*., 2013; Astuti *et al.*, 2015). So, *B. subtilis* has a significant effect on meat quality by reducing fat, increasing meat content, increasing redness and freshness of meat. Lactic acid probiotics are the first generation of probiotics in market and *Bacillus subtilis* increases the growth of these lactic acid bacteria like *Lactobacilli* and *Bifido bacterium* which then helps in secreting lactic acid and acidifies the environment to kill anxious pathogens which can infect chicks (Ohh, 2011). These properties of *B. subtilis* as well as ability to survive harsh conditions make it a suitable candidate for probiotic preparation (Nicholson, 2002). Therefore, the present project is designed to optimize the cultural conditions required for the maximum sporulation of *B. subtilis*.

MATERIALS AND METHODS

Microbial culture

B. subtilis strain was obtained from the IIB, GCU Lahore. The culture was maintained on the nutrient broth (NB) agar medium (nutrient broth 0.8% and agar 2%). The culture was streaked on the Petri plates containing NB agar to obtain single-cell colonies (Haq *et al*., 2012).

Culturing

A single-cell colony of the strain from a freshly streaked plate was used for the development of inoculum. The colony was transferred in the flask containing 10 mL of sterilized NB. The flask was placed in a shaking incubator for growth at 30°C. Overnight grown inoculum (1%, v/w) was transferred to a flask containing sterile 50 mL NB medium followed by incubation for 24-48 hours in a shaking incubator at 30°C.

Determination of sporulation time

Microscopy was performed to determine the sporulation time. Culture samples were withdrawn aseptically from the culture at regular intervals of time and observed under a microscope for estimation of sporulation time of *Bacillus* strain.

Gram staining and spore staining

Gram staining was performed according to the method of Smith and Hussey (2005). For spore staining, Schaeffer-Fulton method was used in order to study the spores of *Bacillus.*

Counting viable number of cells

Viable cell count was performed through the serial dilution and spread plate method (Willey *et al*., 2014). First of all, prepared the 50 mL NB medium in a 250 mL flask and then autoclaved the flasks at 121°C and 15 Psi for 15 minutes. After autoclaving, inoculated the flask in a laminar airflow cabinet and incubated at 37°C and 150 rpm for 12 to 20 hours in a shaking incubator. After incubation, observed the optical density at different time intervals using UV-VIS Spectrophotometer at 600 nm. At the desired OD, prepared specific dilutions 10^{-10} to 10^{-20} and spread 0.1 mL of appropriate dilutions on the individual NB agar plates and incubated these plates at 37°C for 24 hours followed by colony counting.

Optimization of parameters

Optimization of the incubation period

For the purpose of optimization of the incubation period, the incubation period was varied from 12-28 hours but other parameters like pH and temperature were kept constant (Murugesan *et al.,* 2014).

Optimization of pH

For the purpose of optimization of pH, varied the pH (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0) and all the other parameters such as incubation period and temperature were kept the constant. pH was maintained with 1 N NaOH or HCl solution (Murugesan *et al.,* 2014).

Optimization of temperature

For the purpose of optimization of temperature, incubation was carried out at three different temperatures 30, 37 and 45°C, while keeping all the other parameters constant (Murugesan *et al.,* 2014).

Acid tolerance

Acid tolerance was determined according to the method of Hmani *et al*. (2017) and colony-forming unit (CFU) were determined to estimate the number of viable bacteria cells of *B. subtilis*.

RESULTS

Optimization of the incubation period for the growth and sporulation of *B. subtilis*

To optimize the incubation period for the growth and sporulation of *B. subtilis*, the culture was incubated up to 28 hours. The plot of optical density as a function of time period showed is shown in figure 4.1. The optimum sporulation time was found to be 22 hours because maximum OD was obtained at this time period. After that, a decrease in OD was observed.

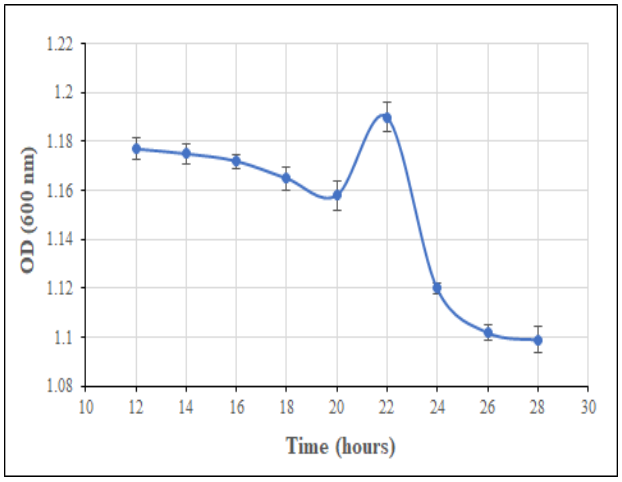

Fig. 4.1: Effect of incubation period on the growth of *B. subtilis*

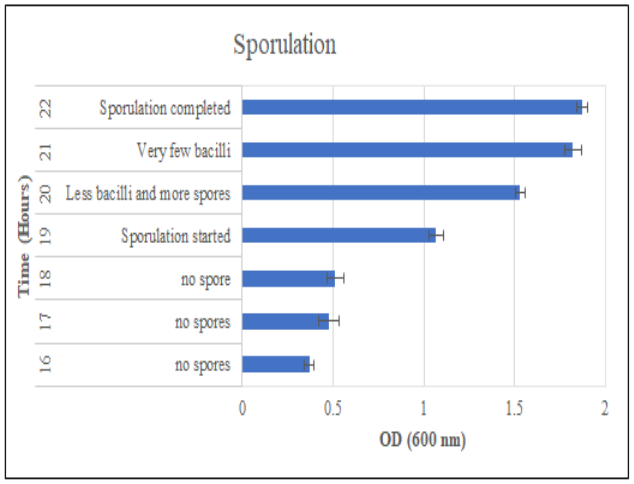

Fig. 4.2: Sporulation of *B. subtilis*

Microscopy was performed at different time intervals to find out the sporulation status and time required for the completion sporulation. The onset of sporulation in culture was observed after 18 hours and the optical density of the culture was 1.068 optical density at that time. Very few bacilli were observed when the culture was 21 hours old (OD 1.822). However, sporulation of the *B. subtilis* completed after 22 hours and OD at this point was 1.872 (figure 4.2). Thus, the sporulation of *B. subtilis* in the present work started after 18 hours and completed at 22 hours. Spore staining was also performed and *B. subtilis* was found to be Gram-positive and subterminal endospore former (figure 4.3).

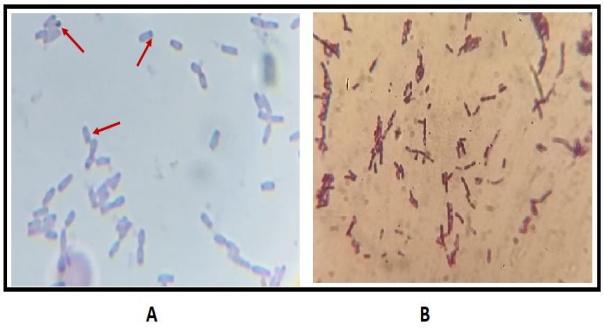

Fig. 4.3: staining of *B. subtilis*
A: subterminal endospores of *B. subtilis*, **B**: gram positive cells of *B. subtilis*

Counting Viable Number of cells

Viable cell counting was performed via serial dilution and spread plate method. The results are shown in Table 1. The viable count increased with OD. When the *B. subtilis* culture was grown for 22 hours, i.e. at the time of completion of the sporulation (OD 1.201), both the OD and the viable count was maximum. The viable count at 22 hours was found to be 8.3 x 10^{23} CFU/ml.

Table I: Viable Cell Counting of *B. subtilis* *

Sr. No.	Time (hours)	OD (600 nm)	CFU/ ml
1.	18	1.107	9.8 x 10^{13}
2.	19	1.130	7.1 x 10^{16}
3.	20	1.158	5.5 x 10^{19}
4.	21	1.161	9.0 x 10^{21}
5.	22	1.201	8.3 x 10^{23}

*CFU = Colony Forming Unit

Optimization of pH for the sporulation of *B. subtilis*

The effect of pH on the sporulation of *B. subtilis* strain was also investigated to find out the optimum pH for the sporulation. For this purpose, pH ranged from 6-9 was tested. This probiotic strain showed the best growth at pH 7.0. The maximum sporulation time was also reduced at the optimum pH and found to be 18 hours, as shown in figure 4.4.

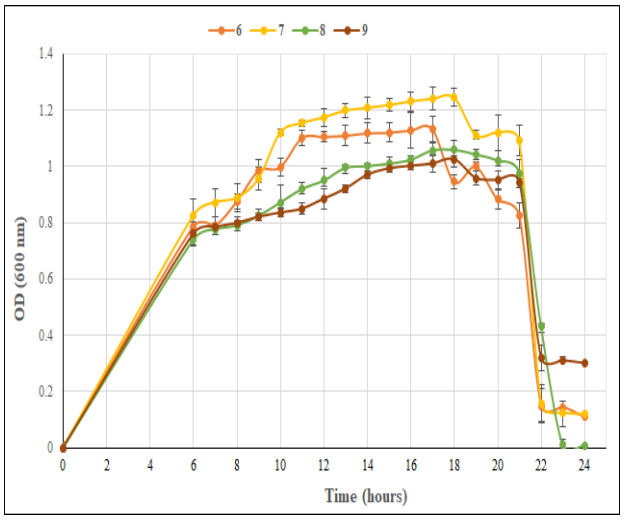

Fig. 4.4: Effect of pH on the sporulation of *B. subtilis*

Effect of temperature on the sporulation of *B. subtilis*

B. subtilis culture was grown at three different temperatures, i.e. 30, 37 and 45°C as shown in figure 4.5. The optimum temperature for this strain was 30°C. All the other temperatures gave comparatively less growth of *Bacillus* as compared to the 30°C.

Acidic tolerance

The acidic tolerance study in acidified NB broth to pH 2. After the growth in acidified NB broth, the culture was spread on the surface of NB agar containing Petri plates followed by incubation at 37°Cfor 24 hours. TVC was found to be 6.4 x 10^{12} CFU/ml.

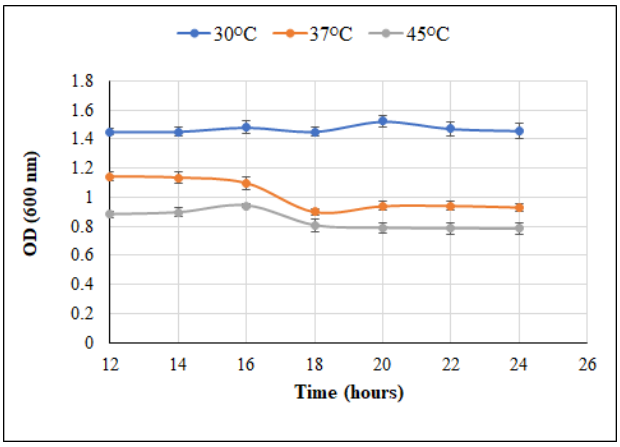

Fig. 4.5: Effect of temperature on the sporulation of *B. subtilis*

DISCUSSION

The probiotics are living microbial feed supplements which help in the host animal by enhancing its intestinal stability. They increase beneficial gut microflora and prevent the colonization of pathogenic bacteria in the gastrointestinal tract. They are also used as a substitute for antibiotic growth promoters in the chick industry. So, the spores of *Bacillus* sp. are mostly used for improving and stimulating the immune system by increasing intestinal microflora of chicks. Their secretions help in maintaining the overall environment of the gastrointestinal tract (Palop *et al.,* 1996; Lee *et al*., 2010; Lefevre *et al*., 2015 & 2017).

Bacteria have to live in a diverse ecosystem and frequently tolerate a large number of fluctuations within a specific environment. The most important way that allows a cell or population to escape from life-threatening situations is the formation of spores (Elizabeth *et al.,* 2014). Sporulation is very important in bacteria because it can preserve the genetical information of bacteria when conditions are not favorable for the normal (vegetative) form of bacteria (Monteiro *et al.,* 2005).

The spore formation requires an appropriate time period for completion of morphological stages and formation of mature spore development, which protects the genome of bacteria against heat, desiccation, radiation, and oxidation (Hoon *et al.,* 2010). In present work, the time for sporulation was expanding from 17 to 22 hours because spores start forming at 17 hours but complete sporulation for *Bacillus* was observed at

22 hours. Einat *et al*. (2011) reported that the sporulation time period for *Bacillus* sp. is 10 hours. Li *et al.* (2019) reported that early or delayed spore formation in *Bacillus* depends on the sporulation medium. In contrast to present work, Korsten & Cook (1996) reported 24 hours as the optimal time for maximum yield of the viable cell.

pH is very important for the development of heat resistant spores through altering the structure of spore (Lee *et al.,* 2003). In the present work, the maximum growth was observed at 7 pH.0. The sporulation time was also reduced to 18 hours at this pH. Many bacteria prefer neutral pH for their optimal growth because high or low pH from this optimal value causes the change in the three-dimensional structure of the cell components. Variations in the 3-D structure destroy the functions of protein (Isnawat & Trimulyono, 2017). Thus, affecting microbial growth and sporulation. Cho *et al.* (2008) also reported that optimal growth for *B. subtilis* was observed at a pH of 7.

Temperature for sporulation is a very important factor that determines the heat resistance of spores and also improves the physical characteristics like OD value and spore wet density (Palop *et al.,* 1999). In the present study, the best growth of the *Bacillus* was observed at 30^{o}C. Temperature exposure is one of the main factors that influence the viability and growth of bacterial cells. In contrast to present work, Satapute *et al.,* (2012) reported the maximum growth of *B. subtilis* at 27°C.

Bacterial spores, when used as probiotics, have to survive acidic conditions in order to reach the small intestine in a viable state. So, acidic tolerance provides a survival challenge against pathogenic organisms in the gastrointestinal tract of animals (Palop *et al.,* 1996). In the present study, the total number of viable cells for *B. subtilis* was 6.4 x 10^{12} CFU/mL at 2 pH. It has been assumed that these spores are able to withstand the harsh environment of the stomach because probiotic bacteria have to bear the acidic conditions of the stomach to reach the colon in a viable state to apply their useful effects. In contrast, to present work, Andriani *et al*. (2017) reported that total plate count for *B. subtilis* was 3.333x10^{4} CFU/ml at 2 pH.

CONCLUSION

In the present experimental study, optimization of sporulation conditions for *B. subtilis* was studied for their use as potential probiotics. Mature spore formation observed after 22 hours and OD at that point was 1.872. The maximum viable count at 22 hours was found to be 8.3 x 10^{23} CFU/ml. The pH of the medium (7) and incubating temperature (30^{o}C) was also optimized. As a result, sporulation time reduced to 18 hours. Acidic tolerance of *B. subtilis* was also determined and viable cell count for *B. subtilis* was found to be 6.4 x 10^{12} CFU/mL at 2 pH. Initial studies show that this strain can withstand the acidic conditions of the stomach, thus it may be proven as a potent probiotic strain for poultry field. However, further studies are required to reduce the sporulation time of this strain prior to test it as a feed supplement for poultry.

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