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Standardization of Various Parameters in the Basal Medium for the Growth of *Bacillus subtilis* Isolated from Soil Near to Ambala region

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ABSTRACT:
To grow and proliferate a microorganism, an efficient medium that enriched with essential nutrients (carbon, nitrogen, minerals, growth factors, vitamins) is prior necessity for a microorganism. A media composition is a crucial part in the field of microbiology that can enhance or decline the bacterial growth. In this study we have isolated *B. subtilis* from wheat soil near to Ambala region. *B. subtilis* is an essential nitrogen fixing bacteria that provides accessible nitrogen in rhizosphere for the better growth of cereals. It improves the biological nitrogen fixation in soil using ecofriendly approach whereas artificial nitrogen approach is an energy demanding process that would be consuming approximately 2.0 % of the world's power by the end of this century. To improve the agricultural sustainability in cereals, it is necessary to optimize the growth of *B. subtilis* using different growth parameters. In the present study, 6 different media composition were chosen to evaluate the maximum proliferation of *B. subtilis* Moreover, different temperature and a pH range from 5 to 9 were selected to reveal the optimum growth and proliferation of *B. subtilis*. During this study, it was concluded that *B. subtilis* showed maximum growth with C1 medium composition (pH 7.0) after incubated at 30º C for 16 hours shaking using UV Spectrophotometer. Furthermore, the optimized medium significantly promoted the growth of *B. subtilis* as compared to Burk's media. Thus, the study would be beneficial for automation of *B. subtilis* for consortia preparation and hence improvement in agricultural sustainability.

Keywords:
Biological Nitrogen Fixation, Diazotrophs, Burk's Media, growth, *B. subtilis*

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INTRODUCTION

Nitrogen is an essential element, necessary for protein and DNA synthesis. Although its wide distribution in the atmosphere, plants have no access for direct utilization of nitrogen from the environment. Only a limited biological form of nitrogen such as nitrate and ammonium are accessible for plant growth and development (Pankievicz et al., 2019). Initially in 20th century, 2 German scientists, Fritz Haber and Carl Bosch, developed a mechanism for industrial-scale nitrogen fixation, or the conversion of dinitrogen into ammonium (Erisman et al., 2008). The introduction of synthetic fertilizers in agriculture was the primary cause in increasing crop productivity *via* Green Revolution, particularly in developing nations. And after that about 50 % of the world's population depends on artificial fertilizers (Heffer et al., 2016). Artificial nitrogen is an energy demanding process in such a way that approximately 2.0 % of the world's power consumption by the end of this century (Bloch et a., 2020; Lassaletta et al., 2014). Furthermore, because of the volatile nature of artificial nitrogen fertilizers in the ground, more than 50 % of the nitrogen fertilizer used annually is lost through runoff which form nitrous oxide a main contributor in global warming (Zhang et al., 2015). To improve the crop productivity in ecofriendly prospects, a major challenge is to enhance the biological form of nitrogen to feed the world's population. Biological nitrogen fixation (BNF) is a promising approach for supply the accessible form of nitrogen for cereal production. In soil, a specific community of diazotrophic bacteria, which use nitrogenase enzyme systems to convert dinitrogen to ammonium, contributes 30 to 50% of total nitrogen in crop lands (Rosenberg et al., 2013). Nitrogenase promotes the reduction of N_2 to NH_3 at optimum condition while the commercial manufacture of fertilizer requires high temperatures and pressure to achieve the synthetic nitrogen (Ladha et al., 2022). Biological Nitrogen Fixation is a microbial based approach where toxic elements degrade into non-toxin products and improves soil productivity (Franche et al., 2009). Irrespective of the phylogenic and geographical nature of diazotrophic microbes and their hosts, a coordination between the microflora and the host crop is required for a successful nitrogen fixation process. This process is an essential as it decreases plant dependency on chemical fertilizers, hence beneficial for agriculture. It has been found that biological nitrogen fixation generates approx. 200 million tons of nitrogen per year at global level (Rosenblueth et al., 2018; Mahmud et al., 2020). In fact, biological nitrogen fixation using diazotrophic bacteria accounts for approximately half of total nitrogen utilized in agriculture land (Puebla et al., 2019). In previous studies, monocot interactions with useful microorganisms particularly epiphytic or endophytic, have received significantly less attention than dicots (Chen et al., 2015). But in present scenario various research groups are working on monocot BNF system to improve the agriculture and crop production. Rhizobium-legume interactions are of great interest because of the bacterial capacity to effectively fix nitrogen within the root nodules. The use of rhizobium in farming reduces the chemical fertilizer, hence minimizing negative consequences, enhancing long-term viability and lowering the price of farming. Cereals are not capable to nodulate with nitrogen fixing bacteria hence it has continuously been a source of dissatisfaction since it restricts the capacity to apply the benefits of BNF to the stable food such as corn, wheat, and paddy (Pankievicz et al., 2021). So, diazotrophs are the finest possibilities for inoculating into crops as biofertilizers in order to prevent the harmful effects of chemical fertilizers. Different microorganisms use different strategies to benefit agricultural crops. They can improve biological nitrogen fixation, phosphate solubilization, and plant growth promotion, or they can have a mixture of these features (Mahanty et al., 2017; Zandi et al., 2016). In addition to solubilizing minerals plants need, such as phosphate, zinc, and potassium, biofertilizers may also fix nitrogen in the atmosphere through the biological nitrogen fixation process and produce biomolecules that encourage plant development, such as hormones. Moreover, biofertilizers can proliferate, take part in the cycling of nutrients, and aid in crop development for sustainable farming when used as seed or soil inoculants (Sun et al., 2020). We have isolated nitrogen-fixing bacteria (*Bacillus subtilis*) from the wheat

soil system from Ambala region using serial dilution procedures on nitrogen-free medium. For optimum growth of *B. subtilis* we have optimized the basic media composition using various parameters such as chemical composition of basic media, pH and Temperature. Other characteristics that affect the growth of *B. subtilis* includes incubation period. The primary goal of the current study is to give a more affordable supply of nitrogen to the agricultural sector and to satisfy crop demands as inflation spreads day by day owing to rising petroleum costs, which impact the pricing of chemical nitrogenous fertilizers. Furthermore, a sustainable agriculture system must use renewable inputs that maximize ecological advantages while minimizing environmental concerns.

MATERIAL AND METHODS

Sub culturing of isolated nitrogen fixing bacterial strain

Nitrogen fixing bacteria *Bacillus subtilis* was collected from the soil system near to Ambala region. Culture was revived in a nitrogen-deficient medium (Burk's), and pure colonies were isolated on Burk's Agar plates. The glycerol stock of isolate was kept at -80^0 C for future use.

Media preparation for optimization of bacterial growth

Different media were used to screen the maximum growth of *Bacillus subtilis* by varying their salt composition and concentration (Mukhtar et al., 2018).

Standardization of bacterial growth using different medium compositions

Bacillus subtilis isolated from wheat soil from Ambala region was optimized for optimum growth using 6 different media. All the above-mentioned media were prepared in separate flasks and autoclaved at 121^0 C for 15 minutes at 15 psi of pressure. Starter culture (10.0 ml) was initially preprepared from isolate colonies and then 1.0 ml of starter culture was used for further bacterial incubation in 250.0 ml flask and experimental work. In this study, Burk's media was set as control as it is nitrogen deficient media. Then the flasks were incubated in shaker at 30^0 C for next 18 hours at 180 rpm. Optical density of each culture was recorded after 18 hours at 600 nm using UV-Spectrophotometer.

Optimization of B. *subtilis* growth at various pH and temperature

From the above prepared media, the medium (C1) which was showing maximum growth of bacterial isolates in standard conditions, is further used to study the effect of different pH and temperature on the bacteria growth. The C1 media with different pH range 5.0, 6.0, 7.0, 8.0 ,9.0 were prepared. Similarly, C1 media with bacterial inoculum were subjected at different temperature range of 25^0 C, 30^0 C, 32^0 C, 37^0 C at rotary shaker with rpm of 160 for 16 hours and again OD was recorded at 600 nm using UV-Spectrophotometer. A blank was maintained in all conditions as control to avoid the contamination.

RESULTS AND DISCUSSION

Bacillus subtilis isolated from wheat soil was grown in 6 different media such as C1, C2, C3, C4, C5, C6 and B1 (Control) as shown in Table. 1. It has been seen that *B. subtilis* was growing in all media but turbidity was different in different media. Optical density (OD) using UV Spectrophotometer shown the similar pattern. During analysis was observed that OD in M2 and M6 media shown least bacterial growth after incubation period of 16 hours at 30^0 C. Moreover, M4, M3 and M5 media showing average bacterial growth under similar growth conditions. Among the 6 different media M1 have shown maximum bacterial growth (Figure 1). M1 composition showing optimum composition for *B. subtilis* growth as compare to other media. To optimize pH and temperature for *B. subtilis* growth, M1 media was chosen as it shows optimum growth among 6 different media. A range of pH from 5.0 to 9.0 and temperature range from 25^0 C to 37^0 C was used for *B. subtilis* growth. Due to presence of an essential salts and minerals in adequate amount in M1 media as per *B. subtilis,* it grown well in respective medium. However, it is reported that magnesium, calcium, iron and sugars play a crucial role in the growth of bacteria (Mukhtar et al., 2018).

Table: 1 Different medium composition for optimization of *B. subtilis*

S. No.	Media	Composition (g/L)
1	C1	Glucose-10.0 gm; K_2HPO_4- 0.64 gm; KH_2PO_4- 0.16 gm; NaCl- 0.2; $MgSO_4$.$7H_2O$- 0.2 gm; $CaSO_4$.$2H_2O$- 0.05; $NaMoO_4$.$2H_2O$- 0.01; $FeSO_4$ - 0.003
2	C2	(NH_2) SO_4 - 0.2; K_2HPO_4 . 0.8; $MgSO_4$ - 0.2; $CaSO_4$ - 0.1; Mo solution- 1ml; $FeSO_4$ - 1ml; mannitol- 20.0.
3	C3	NaCl- 0.4; $MgSO_4$.$7H_2O$- 0.4; KH_2 PO_4 - 0.16; K_2HPO_4 - 0.64; $CaCl_2$ - 0.084; $NaMoO_4$.$2H_2O$- 0.002; H_3BO_4 - 0.003; $FeSO_4$.$7H_2$ O- 0.006; $CoSO_4$ -0.012; $CuSO_4$.$5H_2$ O- 0.0001; $ZnSO_4$.$7H_2O$- 0.012; Sucrose- 40
4	C4	K_2HPO_4 - 0.2; $MgSO_4$ - 0.2; $CaCl_2$ - 0.2; $FeCl_3$ - 0.05 ml of 10%; $NaMoO_4$ - a trace; Manitol- 15.0
5	C5	Extract- 3.0; NaCl- 8.0; Peptone-5.0
6	C6	Glucose- 20.0, $MgSO_4$ .7H2 O- 0.5; K_2HPO_4 - 0.2; $CaCl_2$ - 0.05; $FeCl_3$.$6H_2$ O- 0.10; $NaMoO_4$.$2H_2$ O- 0.05
7	Burk's	0.800 g K_2HPO_4, 0.2 g KH_2PO_4, 0.2 g $MgSO_4$.$7H_2O$, 0.130 g $CaCl_2$, 0.000253 g Na_2MoO_4, 0.00145 g $FeCl_3$ and 20.00 g sucrose

Medium	O. D
C1	0.664
C2	0.111
C3	0.223
C4	0.215
C5	0.373
C6	0.163
BURK's	0.401
BLANK	0.01

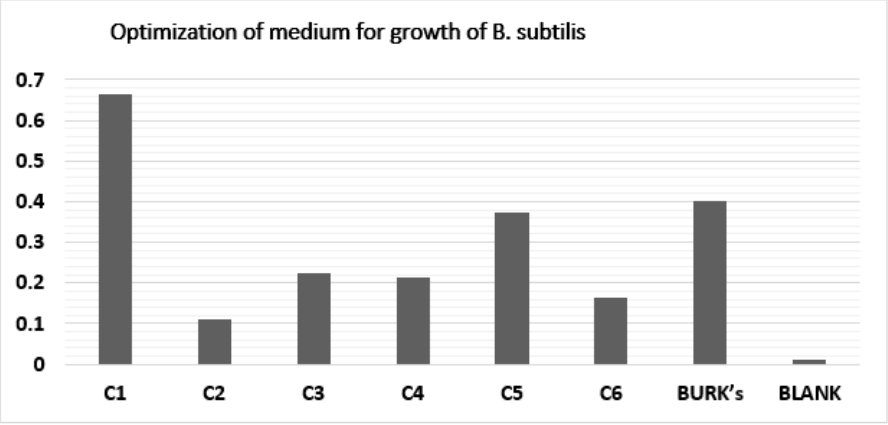

Figure 1: Showing optical density and graphical representation of *B. subtilis* growth in different media composition in comparison to blank and Burk's media.

pH is another prominent factor of soil which influence the proliferation and colonization of plant growth promoting bacteria. For this, a range of pH from 5.0 to 9.0 was selected to evaluate the best pH for *B. subtilis* growth. C1 medium having different pH were used for bacterial inoculum (Figure 3). And it has been observed that maximum growth of *B. subtilis* at pH 7.0 followed by pH 8.0 and pH 9.0 (Figure 2). Very less growth was observed below pH 7.0. It is reported that pH lies between 7.0 to 8.5 or (neutral to slightly alkaline pH) supported maximum population of nitrogen fixing bacterial species. (Ninawel et al., 1997). Below 7.0 pH leads to acidification of media which is detrimental for the growth of bacteria (Rilling et al., 2019). More acidic conditions also suppress the activity of nitrogenase enzyme which is responsible for biological nitrogen fixation in *Bacillus subtilis*. However, a particular pH is mandatory for the functioning of BNF (Ferreira et al., 2016).

TEMP	M1	BURK's
25°C	0.461	0.18
30°C	0.664	0.401
32°C	0.682	0.374
37°C	0.605	0.325

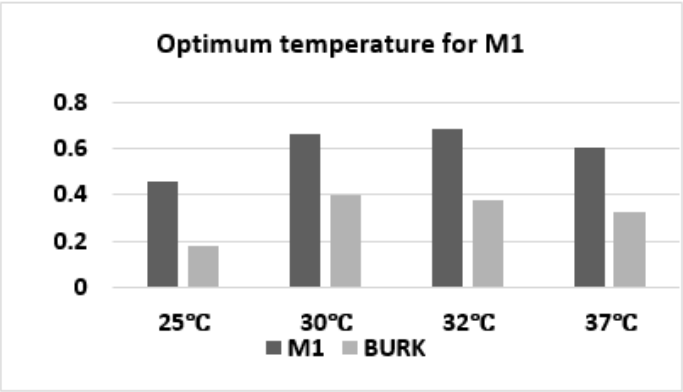

Figure 2: Tabular form of optical density and graphical representation obtained from *B. subtilis* culture grown in M1 at different temperature in comparison to Burk's media.

To optimize the suitable temperature for the growth of *Bacillus subtilis*, a range of temperature were established from 25° C to 37° C. At different temperature, each bacterial species behaves differently. Their survival rate and functioning depend on their capacity to adjust in changing environmental conditions (Bakhshandeh et al., 2014). For evaluate the suitable growth of *B. subtilis, 5* different flasks containing C1 media plus *B. subtilis (inoculum)* were incubated in shaker at 25° C, 30° C, 32° C and 37° C for next 16 hours. Using UV spectrophotometer, 30° C is the most suitable temperature for the growth of *B. subtilis* while at 25° C least growth of *B. subtilis* was observed (Figure 3). It is found that with slightly above and below 30° C bacterial growth was decreased. There is an inhibitory growth effect below 25° C and above 37° C shown by maximum species of bacterial community. However, an optimum temperature is required for bacterial enzymatic and metabolic activities (Torabian et al., 2019; Vats et al., 2021). This study is highly emphasizing the suitable growth of *B. subtilis* under different growth parameters *in vitro*. *B. subtilis* is a nitrogen fixing bacteria which is naturally present in soil near to Ambala region specially in wheat soil. The study could be beneficial for the preparation of biofertilizers to improve the production of wheat crop as well as agricultural sustainability. Thus, the study would be beneficial for eliminating the chemical fertilizers and ultimately enhance crop production.

pH	M1	BURK's
pH 5	0.12	0.186
pH 6	0.415	0.295
pH 7	0.664	0.401
pH 8	0.645	0.377
pH 9	0.621	0.349

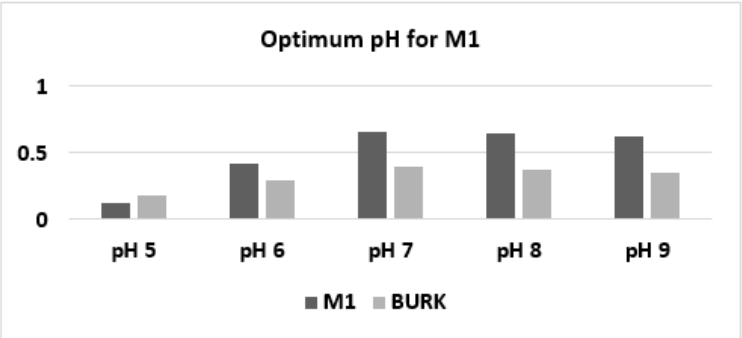

Figure 3: Optical density and graphical representation from *B. subtilis* culture grown in M1 medium having different pH in comparison to Burk's media.

CONCLUSION

Bacterial growth optimization studies are being done to extract the appropriate benefits from the bacterial culture against abiotic stress in agriculture. This is an efficient way to optimize the enhancement of bacterial growth under unfavorable environmental conditions. It could be a useful approach in the manufacturing of biofertilizers which will reduce the stress of toxic and synthetic nitrogen on agricultural land. In the current study, standardization of *B. subtilis* was done to explore the optimum conditions for its colonization. Using different growth parameters such as temperatures, pH and media composition, we described an appropriate growth condition for *B. subtilis*. Since *B. subtilis* is a diazotroph in nature, its efficiency can be improved by using optimized growth conditions to promote sustainable production in agriculture. Due to its ecofriendly and cost-effective behavior this method will be helpful in biological nitrogen fixation of non-leguminous crops which is one of the global challenges. Thus, the study would be beneficial for improved growth of *B. subtilis* towards agriculture and environment sustainability.

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