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Process Development for High Density Cultivation Yield for *Bacillus Subtilis*

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Abstract: ***Bacillus subtilis*** **is an aerobic, endospore forming, gram positive, and catalase positive bacterium.** ***Bacillus subtilis*** **is popular worldwide and have been extensively studied in medicine, biomaterials and agriculture sectors. In agriculture** ***Bacillus subtilis*** **is used on plants as a fungicide. This bacteria form rough biofilm which is beneficial for plant to allow for the control of plant pathogen infection.** ***Bacillus subtilis*** **are produce by fermentation technology. By conventional mode of fermentation process there is limitation to produce biomass. Currently, the demand of** ***Bacillus subtilis*** **is generated by its valuable biomass used in an agriculture sector. Therefore it is need to develop high cell density biomass to enhance production. The process development for** ***Bacillus subtilis*** **was carried in 10 L bench top lab scale glass fermenter with three different cultivation approaches. Batch fermentation process with maintained pH give up highest OD 30.4 mL-1 (against 21.85 in Shake flask and 26.2 in fermentation batch-1) with 100% sporulation efficiency.**

Keywords**:** ***Bacillus subtilis*****, scale up process, sporulation efficiency, high cell biomass, Bio control agent**

1. Introduction

The pathogenic microorganisms affecting plant health is foremost and chronic threats to sustainable ecosystem stability and food production worldwide. Now a day synthetic chemicals are widely used to control these microorganisms. But to continuous use of pesticides has caused environmental problems. Hence to overcome this problem biological disease control using beneficial antagonist is environmentally sustainable alternative to using synthetic pesticides [1]. *Bacillus subtilis* is one of the most promising microorganisms for sustainable agriculture [2] which has been reported as fungicide [3], [4], [5], [6], [7], [8], growth promoter [9], [10], [11], [12], [13], [14] and antagonistic to variety of pathogens [15], [16], [17], [18], [19] in vitro and in vivo studies.

Bacillus subtilis is remarkably diverse bacterial species which is capable of growth within many environments [20]. It can be found in terrestrial and aquatic environments and to survive extreme environmental condition of temperature (30 ºC to 50ºC), pH (5-9) and desiccation [21]. *Bacillus subtilis* is also known as "Soil Dweller" because soil is suitable for spore germination and proliferation [20]. Bacillus subtilis has excellent physiological characteristics. It is aerobic, rod shape (4-2 µm long and 0.25-1.0 µm in diameter), gram positive, catalase positive and endospore forming soil bacterium. Due to its highly acquiescent to genetic exploitation it has become widely adopted as a model organism for laboratory studies. *Bacillus subtilis* is produce through fermentation process in industries. Currently, the demand of *Bacillus subtilis* is generated by its valuable biomass used in a agriculture, medicine and biomaterial sector. Therefore it is need to develop high cell density biomass to enhance production. *Bacillus subtilis* has been highly adaptable metabolism hence it make easy to cultivate on cheap substrate [22]. It grows fast and has a good genetic stability [7]. Due to its simplicity of isolating, handling and maintenance, nonpathogenic nature and extreme metabolic diversity it is used in agriculture sector. *Bacillus subtilis* has a single cell membrane facilitates proteins and enzymes secretions which simplifies downstream processing as well as reduces the process cost. This species is generally recognized as safe (GRAS) [23].

Plants encounter many biotic agents and induce biotic stress in their hosts by disrupting normal metabolism and shown result, limit plant growth and cause of plant mortality [7], [24]. *Bacillus subtilis* is valuable to plants and execute the role as chemical fertilizers and pesticides, acting as a biopesticides, fungicide and biofertilizer. It can be significantly enhance plant growth and represent a mutually helpful plant microbe interaction. *Bacillus subtilis* show evidence of direct biocontrol mechanism includes synthesis of secondary metabolites hormones, antioxidants and cell wall degrading enzymes which assist the plant in its defense against pathogen attack. In indirect biocontrol mechanism *Bacillus subtilis* stimulate of plant growth and induce acquired systematic resistance [25]. Other than this *Bacillus subtilis* can play important role as P solubilizer [26], [27], [28], nitrogen fixer [29] and siderophore producer [30], [31] also they enhance stress tolerance [32], [33], [34]. For plant protection its role as a root colonizer, biocontrol agent, able to protect cortex and vascular tissues of plants,

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increase crop productivity under conditions of biotic and abiotic stress. At present, increasing the *Bacillus subtilis* productivity is the most important challenge in agricultural industries. By conventional mode of fermentation process it is not possible because there is limitation to produce biomass. For the commercialization it is necessary for designing a fermentation process to produce *Bacillus subtilis* biomass to fulfill the above criteria. In industrial settings for production of biomass batch process and fed batch process are common approaches used. Thus, the aim of present research work was to developed process for high density cultivation yield of *Bacillus subtilis* for agriculture use purpose.

2. Material and Methods

1) Microorganism

To develop the high density of biomass *Bacillus subtilis* strain was used for present investigation. The strain was kindly provided by Rajvi enterprises, Ahmedabad, Gujarat, India.

2) Media and culture cultivation condition

Bacillus subtilis strain was grown on nutrient agar petriplates (composition, g L-1: peptone – 5.0, sodium chloride – 5.0, HM peptone B# – 1.5, yeast extract – 1.5, agar – 15) and incubated at 37ºC for 48 – 72 h and maintained at 4 ºC and sub cultured at four weeks intervals. All chemicals and reagents used for present investigation were obtained from HiMedia and Merck, India. The characteristics of *Bacillus subtilis* strain was carried out by routine bacteriological methods by examining the colony morphology, Gram staining, motility test and biochemical tests.

3) Glycerol stock preparation

The inoculum was prepared by transferring a loopful culture from petriplate to a 100 mL Erlenmeyer flask containing 20 mL sterile nutrient medium (composition g L-1: peptone – 5.0, sodium chloride – 5.0, meat extract B# – 1.5, yeast extract – 1.5) at pH 7.3 ± 0.2 and incubated on orbital shaker (CIS-24 plus REMI) at 37 ºC at 160 rpm for 20 h. Glycerol stock was prepared by adding 40% glycerol and stored at - 20 ºC until they used.

4) Shake flask cultivation

All shake flask experiments were carried out in shaking incubator and set to 37 ºC and 160 rpm. For Preseed I stage, 20 mL of seed medium (composition g L-1: tryptone – 17, soya peptone – 3, sodium chloride – 5, dipotassium hydrogen phosphate – 2.0, glucose – 2.5) were inoculated in 100 mL Erlenmeyer flask and incubated for 6-8 h. Preseed I stage was used to inoculate Preseed II stage to an OD 0.5±0.2 per mL-1 and incubated for 6-8 h. After incubation at 37ºC, the suspension was transferred into 100 mL of production medium (composition g L-1: magnesium sulfate –0.25, manganese sulfate – 0.1, calcium chloride – 0.5, corn steep powder – 6, peptone – 5, glucose – 1) in 500 mL Erlenmeyer flask to an OD_{600} of 1.5-2.0 per mL-1. Subsequently, sample was taken every 3 h during cultivation process.

5) Bioreactor fermentation cultivation

Prepared glycerol stock of *Bacillus subtilis* strain was used to inoculate seed medium. Preseed I stage was inoculated for 6-8 h at 37 ºC and 160 rpm. Then next this bacterial strain suspension was used to inoculate in Preseed II stage to an OD 600nm of 1.5±0.2 and cultivated again 6-8 h at 37 ºC and 160 rpm and actively growing cells were cultivated for fermentation inoculum. The details of the inoculum and parameters of Preseed and seed stage are listed in Table – 1.

6) Fermentation process

Fermentations were carried out in the production medium

Table 1
Preseed I and Preseed II stage preparation and parameters

S.No.	Parameters	Preseed stage Details	Preseed stage Details
1	Total volume	20 mL	100 mL
2	Incubation conditions Incubation time Incubation temperature Rotational speed	 6-8 h 37 ºC 160 rpm	 6-8 h 37 ºC 160 rpm
3	pH Initial pH pH at transfer	 7.5 ± 0.2 6.5 ± 0.2	 7.5 ± 0.2 6.5 ± 0.2
4	Culture morphology	Gram positive rods	Gram positive rods
5	Inoculum volume	2%	2%
6	OD at A_{600nm} at seed transfer	0.6 – 0.8 per mL^{-1}	1.5-2.0 per mL^{-1}

Table 2
Fermenter parameters for cultivation of *Bacillus subtilis*

S.No	Parameters	Details
1	Inoculum volume	2 %
2	Fermentation medium volume	6 L
3	Batch parameter Batch time Incubation temperature Agitator speed	 24 h 37 ºC 300 rpm
4	pH Production medium pH	 7.5 ± 0.2 at the time of inoculation
5	% DO	Maintained during batch 60 – 70%
6	Aeration	1.0 vvm
7	Sampling	Every 3 h

Table 3
Biochemical characteristics of *Bacillus subtilis*

S.No.	Test	Results
1	Colony morphology	White, large, circular, adherent colonies with membranous growth.
2	Motility	Motile
3	Spore	Endospore
4	Gram staining	Positive, short rods, thick
5	Catalase	Positive
6	Oxidase	Negative
7	Glucose	Positive
8	Maltose	Positive
9	Fructose	Positive
10	Mannitol	Positive
11	Indole	Negative
12	Starch hydrolysis	Positive
13	Gelatin hydrolysis	Positive
14	Nitrate reduction	Positive
15	Urease	Negative
16	Voges Proskauer	Positive
17	MacConkey	Negative
18	Inositol	Positive

using 10 L bench top lab scale glass fermenter equipped with pH probe. The water sterilization, empty vessel sterilization, medium sterilization was carried out in an autoclave (Omkar Instruments) at 121 ºC (15 psi) for 30 min. Two percent inoculum was transferred into 6 L of production medium. Agitation was provided by Ruston disc turbine with vertical blade disc impellers. To evaluate the cultivation process several parameters temperature and agitation were checked online. P^H monitored using pH electrode (CS-24+ Toscon-Toshniwal), optical density (OD) monitored using UV spectroscopy (UV-1800 Simadzu), sterility, morphology and sporulation efficiency were checked by using microscope (CX 21i, Olympus) offline. Table – 2 represents fermentation process condition for *Bacillus subtilis*.

3. Results

1) Colony morphology and biochemical characteristics

Bacillus subtilis are versatile in their adaptability to the environment. The colony morphology and microscopic characteristics are shown in Figure-1 A and B. When *Bacillus subtilis* cultivated on nutrient agar the morphology shows large, circular, white, rough, flat and opaque colony. Under microscopic observation with staining it shows gram positive violet rods. The biochemical characteristics of *Bacillus subtilis* strain is shown in Table-3.

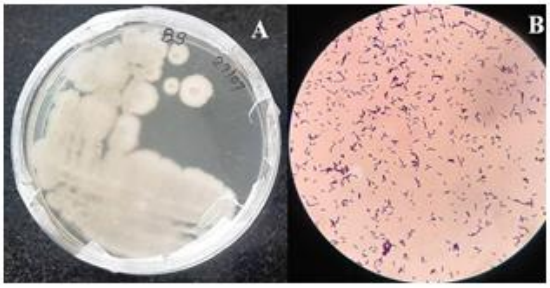

Fig. 1. A) Colony morphology of *Bacillus subtilis* in nutrient agar. B) Bacillus subtilis showing gram positive violet rods in Gram staining

2) Shake flask cultivation

Shake flask cultivation experiment of *Bacillus subtilis* was carried out in production medium shown in Figure-2. The primary aim of this experiment was to assess how *Bacillus subtilis* strain grows in flask, how much time taken for sporulation and how much biomass may be obtained before cultivation in fermenter. The growth curve of *Bacillus subtilis* is shown when grown in production medium in flask optical density and pH parameters measured during course of biomass cultivation experiment. Average growth rate of *Bacillus subtilis* is 3.39 h-1 has been recorded during experiment. A maximum OD_{600}nm 21.85 was reached after 27 h of cultivation. It can be noted from the above graph, pH value dropped from 7.3 to 6.8 from batch starting hour to 15 h. Approximately 18 h pH started increasing gradually and reached up to 8.06 at 48 h. Further, observed the spore initiated after 24 h and 95 % sporulation efficiency seen at 48 h. Enhanced culture growth in production medium could be attributed to availability of C / N ratio and nutrients in adequate quantities.

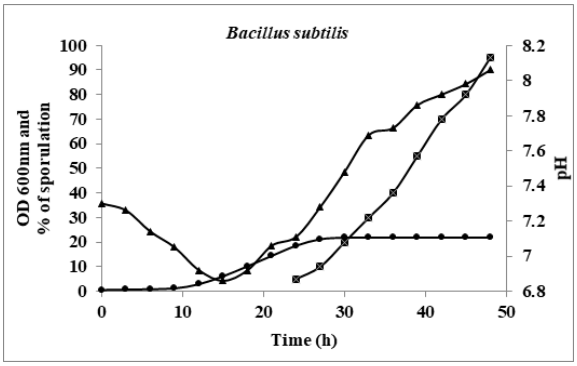

Fig. 2. *Bacillus subtilis* cultivation in shake flask. Plotted are optical density (black circles) pH (black triangle) and % of sporulation (black cross) over time.

3) Batch fermentation

In this experiment, batch fermentation process was conducted to investigate the production of high cell biomass of *Bacillus subtilis* strain. Inoculum was prepared in two stages as mentioned in Table-1. 2% inoculum was used for batch fermentation. Two batches were taken for high cell density biomass.

4) Batch-1

First batch was operated without regulating the pH. The aim of this batch was how the culture grows in fermenter and how much biomass and sporulation was obtained before optimization batch protocol. The parameters of batch were set as temperature 37ºC, agitation 300 rpm, air 1vvm and back pressure 0.3 bars. The results are shown in Figure-3.

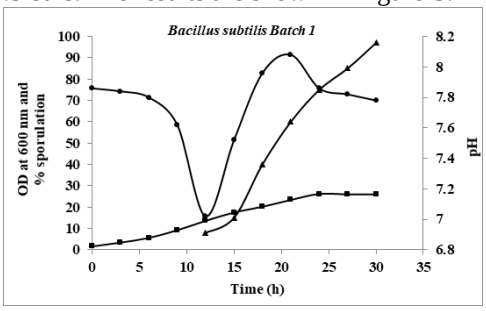

Fig. 3. *Bacillus subtilis* cultivation in fermenter. Plotted are optical density (black square), pH (black circles) and % of sporulation (black triangle) over time.

As compared to shake flask batch the duration of the fermenter batch 1 was reduced by 18 h. Highest OD (26.2) was achieved at 24 h. On expanding the batch further than 24 h the OD was starting declining so the batch was harvested at 30 h from the starting of production. It can be assumed that production in fermenter OD was highly improved because agitation and aeration conditions as compared to shake flask batch. Sporulation efficiency (97%) also increased in Batch-2 than shake flask batch. The sporulation started after 12 h. It can be noted from the above graph, pH value dropped from 7.8 to 7.0 from batch starting hour to 12 h. approximately, from 15 h pH started increasing gradually and reached up to 7.7 at 30 h. These results are motivating because reduction in time and progress seen in sporulation efficiency. Thus process becomes time consuming and more economical. To cultivate the high

density biomass with 100% sporulation efficiency could be to maintain the pH in fermentation batch. Therefore, second batch was decided to achieve this goal with maintaining pH.

5) Batch-2

This experiment was conducted fermentation batch where pH was maintained. The trend of OD, pH and % sporulation efficiency are presented in Figure-4.

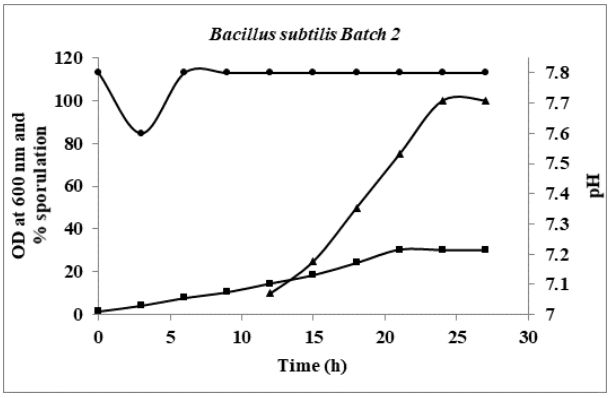

Fig. 4. *Bacillus subtilis* cultivation in fermenter with maintaining pH. Plotted are optical density (black square) pH (black circles) and % of sporulation (black triangle) over time.

Batch 2 was harvested at 27 h much before than shake flask batch and fermentation Batch 1. As compared to previous batch this batch was reduced by 6 h. On expanding the batch further than 21 h the OD was starting declining so the batch was harvested at 27 h from the starting of production. Highest OD (30.4) was reached at 24 h. When pH value dropped from 7.8 to 7.6 in first 3 h it started to maintain with 25% ammonia and 20% orthophosphoric acid (OPA) up to 100% sporulation efficiency achieved. From a graph it can be shown that when pH maintained in fermentation batch OD was highly improved. Sporulation efficiency (100 %) was achieved at 24 h. The sporulation started after 12 h of fermentation batch.

4. Discussion

For sustainable agriculture plant disease is control by biological agents. *Bacillus subtilis* reveals clues for plant growth promotion and Biocontrol agents [1], [2]. The physiological and chemical character showed *Bacillus subtilis* is a universal cell factory for agriculture industry. It is a versatile weapon for plant pathogen [4]. For successful cultivation of biomass suitable environmental conditions are required and it get desired product. Factors affecting on biomass have continuously monitored by [35]. Average growth rate of *Bacillus subtilis* is 3.39 h-1obtained in shake flask experiments, whereas maximum OD 30.4 mL-1 was obtained in fermentation batch 2. The use of batch fermentation with maintain pH in the industry it takes advantage and produce high cell density biomass for agriculture purpose. Batch cultivations showed that *Bacillus subtilis* is promising bacterial culture increased production and sporulation efficiency. According to [36], high cell density was first carried out with yeasts for single cell proteins, ethanol and biomass. We have shown the biomass of *Bacillus subtilis* increased in fermentation batch by maintaining the pH by performing shake flask and fermenter batch and their analysis. The dynamics of colony growth and its environment pH dependence were clarified from our experiments. The cultivation of strain in fermenter batch confirmed that the superior production and high cell density biomass achieved. Such spore production result shown by [37], [38]. [5], have reported *Bacillus subtilis* impacted as a fungicide. [39], have reported optimization of nutrient feeding was developed to improve the growth in fed batch fermentation and get maximum cell biomass in culture broth in a 5 L fermenter. The pH of living cell is one of the most important physiological parameter. Monitoring the pH inside of *Bacillus subtilis* during various stages of its life cycle and expressed that during exponential growth, early and late sporulation, spore germination and subsequent spore outgrowth are depends on pH [40]. In our experiments the trend of OD and % sporulation efficiency was observed in increasing over time. Same trend are reported by [2], [15], [22], [35]. In agriculture sector the product interested in carrier base with biomass due to its self life. Hence sporulation efficiency is important. We here reported the high cell density biomass, sporulation efficiency are increase with maintaining the pH. 100% sporulation was seen in batch-2 at 24 h.

5. Conclusion

For agriculture purpose a high cell density process for *Bacillus subtilis* was successfully demonstrated in a 10 L bench top glass fermenter with cost effective medium sequential chances in process. Our batch fermentation strategy with maintaining pH level was worked and increase in the productivity of *Bacillus subtilis* to 30.4 OD and 100 % sporulation efficiency at 24 h. Scale up the process is valuable contribution for high density biomass which make a cost effective, ecofriendly for agriculture sector.

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