

Development of Cost-effective Homemade Basal Medium for Culturing *Bacillus subtilis* strain KPA *In Vitro*

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Abstract

The present preliminary context was investigated to formulate a cost-effective homemade basal medium for the successful growth and subsequent culturing of *Bacillus subtilis* strain KPA *in vitro*. In view of this, PSC (Potato Soya chunk Chickpea) agar medium was prepared by adding aqueous filtered solutions of potatoes (*Solanum tuberosum*), soya chunks (*Glycine max*) and Chickpeas (*Cicer arietinum*) in definite proportion. This newly prepared medium favoured the growth of strain KPA and showed successful culturing after 24 h of incubation. The results observed on the agar plates were more or less similar to the growth of strain KPA on nutrient agar (NA) plates. Most importantly, the isolate grown into PSC medium showed more or less similar pattern of growth profile as in NB medium. Briefly, PSC agar medium can be used *in vitro* for the subsequent culturing of *B. subtilis* strain KPA in a cost-effective manner.

Keywords: *B. subtilis*, Growth; Homemade medium; PSC agar medium

Introduction

Fermentation media play a very important role not only in the successful growth and culturing of bacteria but also enhancing the production of bioactive components for their industrial applications [1]. The optimization of bacterial growth depends upon the medium constituents such as carbon sources, nitrogen sources, vitamins, pH etc. The specific nutrients supplied into the production medium contribute towards the maximum biomass yield. Carbon is the essential element for the growth and metabolism of bacteria that has profound influence on the bacterial biomass. Similarly, nitrogen sources are important variables, influencing the growth of bacterium. pH and incubation temperature are other valuable factors that have profound effect on the growth of bacteria.

Bacillus sp. is widely distributed in the natural environments and inhabits a variety of extreme and contaminated environments [2]. It constitutes a large, heterogeneous group of gram positive, aerobic, endospore-forming, and motile with peritrichous flagella and rod-shaped bacteria [3]. At present, *B. subtilis* is the most important industrial microorganism that has been exploited commercially due to short fermentation cycle, safe handling and potentiality to secrete secondary metabolites extracellular into the medium [4].

The growth of *B. subtilis* as well as production of bioactive components from this bacterium is dependent upon the medium constituents. There is a continuous effort by researchers to optimize different variables of fermentation medium in order to maximize the bacterial growth. In this regard, cost of the media plays a major role that may interrupt the research work due to financial issue. Therefore, in the light of growing *B. subtilis* for various research purposes, we had undertaken a preliminary step to find a suitable and inexpensive basal media that may allow the growth of bacterium *in vitro* more or less

similar to the commonly available undefined media such as Nutrient broth.

Materials and Methods

Isolation and identification of bacterium

Poultry faeces soil sample was collected from poultry farm of Guduvanchery, Tamil Nadu (India). The sample was serially diluted and 0.1 ml of aliquots was spread over NA plates. The plate was incubated at 37°C for 24 h. Further, the isolate was purified by repeated streaking on NA plates and preserved in slants at 4 ± 2°C. Purified isolate was characterized by morphological as well as biochemical analysis according to the Bergey's Manual of Systemic Bacteriology [5].

The 16S ribosomal RNA was amplified by using the PCR (ependorfep.Gradient) with Taq DNA polymerase and primers 27F (5' AGTTTGATCCTGGCTCAG3') and 1492R (5' ACGGCTACC TTGTTACGACTT 3'). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 52°C for 1 min and primer extension at 72°C for 1 min. At the end of the cycling, the reaction mixture was held at 72°C for 10 min and then cooled to 4°C. The amplicon was sequenced by an automated sequencer (Genetic Analyzer 3130, Applied Biosystems, USA) and sequences were submitted to NCBI, Genbank.

Preparation of PSC (Potato Soya chunk Chickpea) basal medium constituents

Fresh potatoes, soya chunks and chickpeas were purchased from the local market of Guduvanchery, Tamil Nadu, India. Potatoes and other ingredients were washed in running tap water. Twenty five grams of soya chunks and chickpeas were soaked in 100 ml of distilled water using two separate sterile beakers for 12-18 h. On the other hand, 3-4 pieces of fresh and disease free potatoes were peeled, cut into small

pieces and mixed with 50 ml of distilled water. After required soaking period, soya chunks, chickpeas and potatoes were ground using sterile mortar and pestle. Later, the distilled water used for soaking was mixed into these ground ingredients again. Subsequently, the peels of chickpeas were removed. Now these ingredients were boiled for 10 min in separate beakers and the respective aqueous layer was collected separately after centrifugation at 2500 rpm for 5 min. These aqueous solutions of each ingredient were sterilized using 0.22 µm membrane filter and stored for media preparation.

Preparation of various agar medium for comparative study

Here, different experimental sets of agar medium were prepared in order to observe the comparative growth of isolate. Further, the growth of isolate on PSC agar plates was compared with the NA plates (g/l- Peptone 5.0, Beef extract 3.0, Yeast extract 2.0, Sodium chloride 5.0, pH 7.0-7.2) as positive control and agar plates as negative control. The following experiments were carried out in triplicate.

Experiment 1 – NA + pH (7.0-7.2) + distilled water (75 ml)

Experiment 2 - Aqueous solutions of P (1% v/v) + S (1% v/v) + C (0.5% v/v) + NaCl (0.25 % w/v) + agar (1.8% w/v) + pH (7.0-7.2) + distilled water (75 ml)

Experiment 3 - Aqueous solutions of P (1% v/v) + S (1% v/v) + C (0.5% v/v) + NaCl (0.25 % w/v) + agar (1.8% w/v) + yeast extract (0.2 % w/v) + pH (7.0-7.2) + distilled water (75 ml)

Experiment 4 - Aqueous solutions of P (1% v/v) + S (1% v/v) + C (0.5% v/v) + NaCl (0.25 % w/v) + agar (1.8% w/v) + beef extract (0.3 % w/v) + pH (7.0-7.2) + distilled water (75 ml)

Experiment 5 - Aqueous solutions of P (1% v/v) + S (1% v/v) + C (0.5% v/v) + NaCl (0.25 % w/v) + agar (1.8% w/v) + peptone (0.5 % w/v) + pH (7.0-7.2) + distilled water (75 ml)

Experiment 6 - NaCl (0.25 % w/v) + agar (1.8% w/v) + yeast extract (0.2 % w/v) + pH (7.0-7.2) + distilled water (75 ml)

Experiment 7 - NaCl (0.25 % w/v)+agar (1.8% w/v)+beef extract (0.3 % w/v)+pH (7.0-7.2) + distilled water (75 ml)

Experiment 8 - NaCl (0.25 % w/v)+agar (1.8% w/v)+peptone (0.5 % w/v)+pH (7.0-7.2) + distilled water (75 ml)

Experiment 9 - NaCl (0.25 % w/v)+agar (1.8% w/v)+aqueous solutions of P (1% v/v)+pH (7.0-7.2)+distilled water (75 ml)

Experiment 10 - NaCl (0.25 % w/v)+agar (1.8% w/v)+aqueous solutions of S (1% v/v) + pH (7.0-7.2)+distilled water (75 ml)

Experiment 11 - NaCl (0.25 % w/v)+agar (1.8% w/v)+aqueous solutions of C (1% v/v) + pH (7.0-7.2)+distilled water (75 ml)

Experiment 12 - Agar (1.8% w/v)+pH (7.0-7.2)+distilled water (75 ml)

(Note- P: Potatoes; S: Soya Chunks; C: Chickpeas)

The bacterial cultures were streaked onto the agar plates containing above mentioned experimental ingredients and incubated at 37°C. Each agar plate was observed for the growth of bacterial cultures after 24 h of incubation and results were interpreted.

Growth profile of strain KPA

Five hundred microlitres of overnight grown strain KPA were inoculated into 50 ml of sterilized NB and PSC media (pH- 7.0). The culture was kept for incubation at 37°C in a rotatory shaker and absorbance was read every 1 h at 600 nm. The growth profile of strain KPA grown in PSC medium was compared with respect to NB medium.

Results and Discussion

Based upon the morphological and biochemical characteristic (Table 1), the isolate was identified as *Bacillus* sp.

Tests	strain KPA
Surface	Smooth
Colony colour	White
Opacity	Opaque
Gram reaction	Gram (+)
Cell shape	Rod
Endospore	+
Motility	+
Glucose test	+
Lactose test	+
Mannitol test	+
Starch hydrolysis	+
Tween 80	+
Casein hydrolysis	+
Indole	-
Methyl Red	-
Voges Proskauer	+
Citrate utilization	+
Urease test	-
Catalase test	+
Oxidase test	+
Aerobic growth	+
ONPG	+
Nitrate reductase	+
Arginine	-
Sucrose	+
Arabinose	+
Trehalose	+
Malonate	+

Pigment	-
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Table 1: Morphological and biochemical characteristics of strain KPA. Note: '+' = Positive; '-' = Negative

The isolate was found to be non-pigmented endospore forming gram positive rod shaped bacteria. Further, the isolate showed potentiality to degrade starch as well as casein. On the other hand negative results were observed for indole, methyl red and urease test. In addition to this, positive results were obtained towards different carbohydrate tests. The isolate was identified as *B. subtilis* strain KPA (Accession number-KC918878) after 16S rRNA gene sequencing and NCBI BLAST.

Strain KPA was able to grow in the newly formulated inexpensive homemade basal medium which constitutes aqueous solutions of P (1% v/v), S (1% v/v), C (0.5% v/v), NaCl (0.25 % w/v), agar (1.8% w/v) and pH (7.0-7.2) (Figure 1B). The growth of bacterium on PSC agar medium was observed more or less similar to the bacterial growth on NA medium (Figure 1A). The supplementation of yeast extract, beef extract and peptone into the PSC agar medium favoured the growth of bacteria (Figures 1C, 1D and 1E). On the other hand incorporation of yeast extract, beef extract and peptone individually with agar also showed the growth of strain KPA (Figures 1F, 1G and 1H). Surprisingly, the supplementation of aqueous solution of P, S and C individually into the respective media showed negative impact on the growth of bacterium (Figures 1I, 1J and 1K). Similarly, the medium containing only agar showed lack of bacterial growth (Figure 1L).

Figure 2 shows the growth profile of strain KPA in the PSC and NB medium. The isolate obtained lag phase after 2 h of inoculation. Strain KPA reached exponential phase after 8 h of inoculation when grown into the NB medium. On the other hand, similar pattern of exponential phase (8-10 h) was observed for strain KPA when grown in PSC medium.

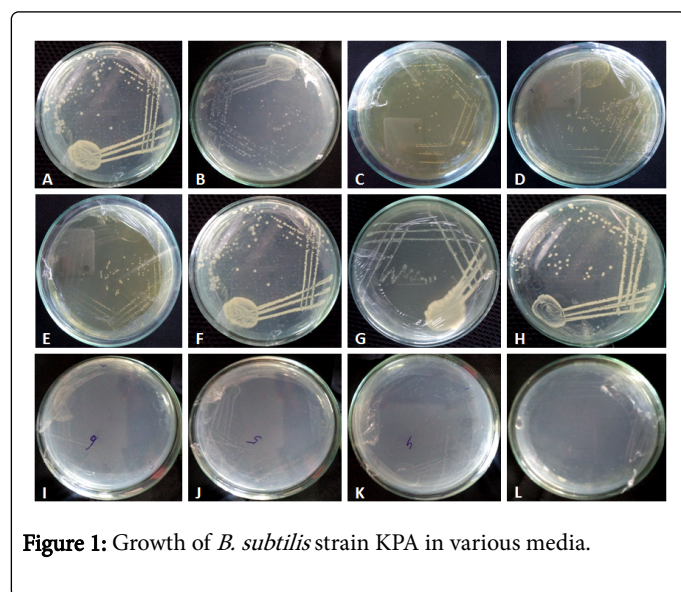


Figure 1: Growth of *B. subtilis* strain KPA in various media.

In the above figure (A) NA, (B) aqueous solutions of P (1% v/v) + S (1% v/v) + C (0.5% v/v) + NaCl (0.25 % w/v) + agar (1.8% w/v), (C) aqueous solutions of P (1% v/v) + S (1% v/v) + C (0.5% v/v) + NaCl (0.25 % w/v) + agar (1.8% w/v) + yeast extract (0.2 % w/v), (D) aqueous solutions of P (1% v/v) + S (1% v/v) + C (0.5% v/v) + NaCl

(0.25 % w/v) + agar (1.8% w/v) + beef extract (0.3 % w/v), (E) aqueous solutions of P (1% v/v) + S (1% v/v) + C (0.5% v/v) + NaCl (0.25 % w/v) + agar (1.8% w/v) + peptone (0.5 % w/v), (F) NaCl (0.25 % w/v) + agar (1.8% w/v) + yeast extract (0.2 % w/v), (G) NaCl (0.25 % w/v) + agar (1.8% w/v) + beef extract (0.3 % w/v), (H) NaCl (0.25 % w/v) + agar (1.8% w/v) + peptone (0.5 % w/v), (I) NaCl (0.25 % w/v) + agar (1.8% w/v) + aqueous solutions of P (1% v/v), (J) NaCl (0.25 % w/v) + agar (1.8% w/v) + aqueous solutions of S (1% v/v), (K) NaCl (0.25 % w/v) + agar (1.8% w/v) + aqueous solutions of C (1% v/v) and (L) agar (1.8% w/v)

(Plate A - Good growth, B - Good growth, C - Good growth, D - Good growth, E - Good growth, F - Good growth, G - Good growth, H - Good growth, I - Poor growth, J - Poor growth, K - Poor growth and L - No growth)

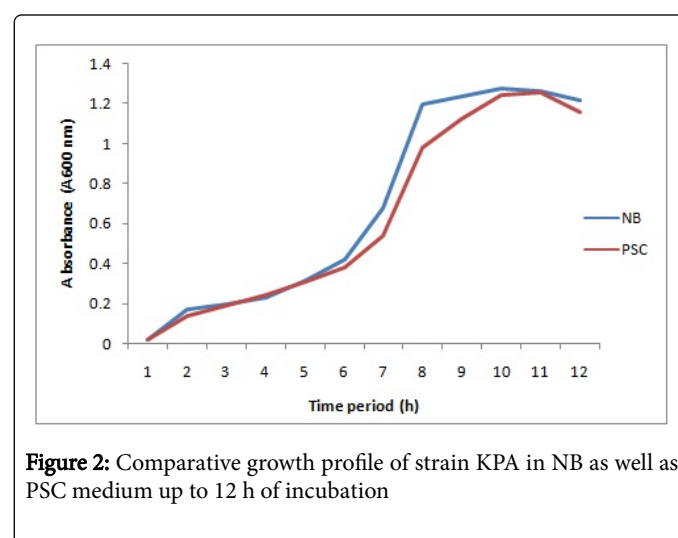


Figure 2: Comparative growth profile of strain KPA in NB as well as PSC medium up to 12 h of incubation

Fermentation medium plays a critical role in maximizing the growth of bacteria [6]. In the present context, the aqueous solutions of potatoes, soya chunks and chickpeas favoured the growth of *B. subtilis* strain KPA. It might be because of the nutritional components, essential elements and growth factors of these ingredients, which are suitable for the growth, as well as subsequent culturing of bacterium *in vitro*. In fact, in this newly formulated homemade media (PSC agar medium), potatoes were used as a source of carbohydrate. On the other hand, soya chunks and chickpeas were incorporated into the medium as vital sources of proteins, carbohydrates and ions.

The study also demonstrated that yeast extract, beef extract and peptone (ingredients of NA medium) favoured the growth and successful culturing of strain KPA if incorporated individually into the medium i.e. the bacterium can be cultured by adding any of these ingredients of NA into fresh agar medium. Further, the lack of bacterial growth and their culturing on separate medium consisting individually aqueous solution of potatoes, soya chunks and chickpeas indicated that a combination of aqueous solution of potatoes, soya chunks and chickpeas in definite proportions are essential for the successful culturing of strain KPA. PSC agar medium is easy to prepare and it showed similar pattern of bacterial growth as observed on NA plates as well as through growth curve analysis.

Conclusion

The present study investigated that PSC is an appropriate basal medium for the successful culturing and growth of *B. subtilis* strain KPA in comparison with commonly used NA medium. The ingredients for the preparation of PSC medium are cheap and easily available that can be prepared for research purposes without any limitations. Additionally, the growth profile of strain KPA on PSC medium was observed more or less similar to that of NB medium. Further study is in progress to formulate the definite proportions of PSC constituents and also to identify the biochemical properties of isolate grown in PSC medium. An extensive study is also required in order to investigate the growth of other industrially important *Bacillus* sp. on PSC agar as well as broth medium.

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