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GROWTH OF *BACILLUS SUBTILIS* AND PRODUCTION OF ACETIC ACID WITH ROTTEN POTATO: USED AS SUBSTRATE

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ABSTRACT: The *Bacillus subtilis* is anaerobic gram-positive, dominant and non-pathogenic bacterial workhorses in industrial fermentations. In present work, *B. subtilis* LC4 cultured on a number of combinations of TY-medium with peels and peeled-off rotten potatoes to investigate its growth and level of secondary metabolites production including acetic acid. Cell culture was harvested after 42 and 62 h and maximum growth rate was observed in LB_5 (peeled off potato extract) and LBo (TY-medium) cultures, while culture OD600 was low at 62h of all. It might be reduced with the shortage of nutrients in the medium that lead to killing of cells. Reducing sugars and total proteins were observed higher ($p \leq 0.05$) but reversed in both harvests to the decreasing rate of cell growth or multiplication rate of all cultures. Similar trend in production of acetic acid was also noted ($p \leq 0.05$) in all cultures. Increasing level of reducing sugars concentration among the culture indicates the cells are growing under nutritional stress. Meanwhile production of acetic acid along the higher concentrations of reducing sugars could be involved to initiate signals for sporogenesis in *Bacillus subtilis* cells. It could be a best adjustment of Bacillus cells for their survival in nutrient medium at stationary or decline growth phase. Both mixture of peels and starch extracts of rotten potato are recommended as the best productive and cheapest substrate for growth of *Bacillus subtilis* as well as may be recommended for microbial based extracellular proteins and acetic acid production.

INTRODUCTION: The members of Bacillus genus including *Bacillus subtilis* are being important for industrial organic compounds productions. Being generally recognized as SAFE (GRAS) gram positive bacteria and able to produce majority of volatile organic substances [1, 2, 3, 4]. Among growth cultures and biotransformation capabilities, *B. subtilis* greatly effect on population of microflora and compounds of nutrient flavours [5, 6].

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Acetic acid is one among most predominant weak organic acid involves in flavoring and antimicrobial organic agent in vinegar industrial products [7, 8]. Acetic acid has many industrial applications with annual production of 2 million tonnes [9]. Mainly used in flavouring as well as preservation of food and beverage industries, while as a cleaning agents in metallurgy and pharmaceuticals. Industrial fermentation is dependent on almost pure sucrose feed-stocks with rising costs of final bio-product and increasing risks over human food security.

However, a number of switches are available with low costs and more sustainable feed-stocks for concerned type of fermentation organism [10, 11]. Under this scenario, *B. subtilis* is capable to survive on diverse plant waste materials to use it as a

carbon sources, while exploitation is needed to locate great potential of underused resource for final bio-production. Fermentation based production of organic acids and proteases is a fast increasing field of research to fulfill food industrial requirements [12, 13]. Utilization of novel microbial cells and agriculture based renewable plant wastes is an emerging technology for economic and cost-effective bio-production of high-valued organic acids [14, 15].

During domestic or industrial usages, potatoes are peeled off, which is almost 15 - 40% potato peel waste (PPW) depends on peeling method [16, 17]. Purpose of potato peeling is that it is not digestible by non-ruminants as being too fibrous [18]. The PPW is an inexpensive and rich with lipids, proteins, starch, polyphenols, lignin and polysaccharides. It is a valuable cheap carbon source of fermentation processes [19, 20, 21].

A number of industrial strains are yielding more than 70% citric acid [22]. Continuous production over many decades by these microbial strains has under gone a number of random mutagenesis. It results into a new unknown strain with instable genome. Meanwhile, *B. subtilis* has been rationally engineered after its complete genome sequencing for genome scaled metabolic modelling [23, 24]. Now its growth handling and genome modelling could make useful under normal to drastic growth conditions for industrial bio-production [25]. In this study, wild type *Bacillus subtilis* is grown on various rotten potato based fermentation cultures in comparison to standard growth medium. Comparative growth rate and production of acetic acid is evaluated on these cultures.

MATERIALS AND METHODS:

Bacterial Strain and Culture Conditions: Pre-culture of wild-type *B. subtilis* LC4 was grown with frozen 16% glycerol stock [26]. Almost 5 µL of stock culture was inoculated in TY (10 g L^{-1} trypton, 5 g L^{-1} NaCl and 5 g L^{-1} yeast extract) medium [27] and incubated at 37 °C under ambient air with 250 rpm orbital shaking for overnight. This pre-culture was inoculated in fresh TY medium and its different combination with rotten potato extracts to develop initial OD600 of approximately 0.01 as in **Table 1**. Each culture was maintained with three replicates.

Rotten Raw Potato Extracts: Rotten potatoes were collected from nearby local market and washed with 70% ethanol. Cleaned potatoes were peel off than weight of peels and peeled off potatoes was recorded. Both were chapped into small pieces. The peels and peeled off potation were grinded with grinder mixer in triple and double volume of distilled water (dH_2O) respectively. The slurry was autoclaved at 121 ºC for 20 min. This homogenized slurry was filtered through muslin-cloth. The filtrate was mixed with TY medium to raise different combinations of growth cultures with variant carbon sources **Table 1**. Volume of each culture was maintained 25 ml. Cultures were harvested at the time interval of 42 and 62 h.

Determination of Growth Rate: The cultures were harvested at 42 h and 62 h after incubation. The OD600 was taken and were centrifuged at 5,000 rpm for 10 min). Cell pellet was frozen and supernatant was store at 4 °C for the estimation of organic substances produced by *Bacillus subtilis*.

Estimation of Total Proteins and Reducing Sugars: Total protein contents in the supernatant were measured by following a method by Lowry *et al*. [28] A mixture of 0.5 ml of sample and 2.5 ml alkaline copper reagent was raised. It was thoroughly mixed and stand at room temperature for 10 min than 0.25 ml Follin-Ciocalteau reagent was added. After 30 min absorbance was read at 750nm. Reducing sugars were also determined in same sample as by Miller [29]. The 1 ml supernatant was mixed with 1 ml 2,6-dinitro salicylic acid (DNS). The mixture was heated for 5 min in boiling H_2O. Absorbance of cold reaction mixture was read at 540 nm.

Estimation of Acetic Acid Production Rate: Supernatant of each culture was subjected to acetic acid production estimation. Titration method was followed as described by Beheshti and Shafiei [30]. Briefly, exact 5 ml of sample was mixed with 20ml dH_2O in 250 ml flask. Almost 5 drops of phenolphthalein (0.1 g phenolphthalein, 40 ml dH_2O, 60 ml ethanol) mixture were added and mixed thoroughly. This mixture was titrated against 0.5N NaOH (200 ml burette) until final pale pink colour was appeared. Amount of acetic acid (g) in 100 ml culture medium was calculated as:

Acetic acid (g/100 ml) = NaOH (ml) volume × 0.03 × 20

Statistical Analysis: Cultures for each medium, three replicates were raised. The collected data from this experiment was subjected in final for comparative data significance analysis at the level of 5% differences. This significance was computed with COSTAT (CoHort software, Berkeley, USA) computer based statistical package.

RESULTS AND DISCUSSION: The *Bacillus subtilis* is aerobic, non-pathogenic bacteria found in soil, water, air and healthy to humus plant and animal debris [31, 32]. A number of *Bacillus species* are useful for a number of industrial applications due to its capability for production of large number of enzymes [33, 34]. A number of bacteria including yeast have history for safe prebiotic consumption [35, 36]. The *B. subtilis* produces antimicrobials and exerts immune stimulation for overall gut microflora enhancement [37, 38]. Also useful in preparation of a large number of consumable fermented foods with numerous health benefits [39, 40]. Even low pH organic acids are being bactericidal including acetic acid [41], while *Bacillus spp*., also produces both lactic and acetic acid and occasionally, butyric acid as it can grow anaerobically in presence of glucose and pyruvate in medium [42, 43].

In present investigation, *B. subtilis* LC4 was cultured on a series of cultures derived from TY-medium with combinations to rotten potatoes for the study of comparative cell growth and acetic acid production by microbial cells. For this purpose a number of parts of rotten potatoes were targeted. In total six cultures of *B. subtilis* were conducted of various cell nutrient medium **Table 1**.

TABLE 1: COMPOSITION OF VARIOUS *B. SUBTILIS* LC4 GROWTH CULTURES SUPPLEMENTED WITH ROTTEN POTATO AS A CARBON SOURCE

S. no	Medium	Composition of medium
1	LB_0	TY medium: 10 g L^{-1} trypton, 5 g L^{-1} NaCl and 5 g L^{-1} yeast extract
2	LB_1	¼ volume TY medium + ¾ volume dH_2O
3	LB_2	¼ volume TY medium + ¼ volume peel extract + ½ volume dH_2O
4	LB_3	¼ volume TY medium + ¼ volume peeled potato extract + ½ volume dH_2O
5	LB_4	¼ volume peel extract + ¾ volume dH_2O
6	LB_5	¼ volume peeled potato extract + ¾ volume dH_2O

Volume of each culture was 25 ml and maintained in 200 ml conical volumetric flask

These cultures were harvested after 42 and 62 h, maximum growth was observed on LB_5 (peeled off potatoes as substrate) than others including control LB_0 (TY-medium) medium. While, decrease in cell multiplication or cell death was observed in all maintained six cultures but high reduction ($p \leq 0.05$) noted in LB_3 (¼ volume TY medium + ¼ volume peeled potato extract + ½ volume dH_2O) and LB_4 (peels) cell cultures **Fig. 1**. Comparatively, peeled off potato extract showed good and stable growth in *B. subtilis* culture than peels off potato and even standard TY-medium. The starch of rotten potato could be used as an economic substrate for the production of large number of industrial important enzymes and other important secondary metabolites.

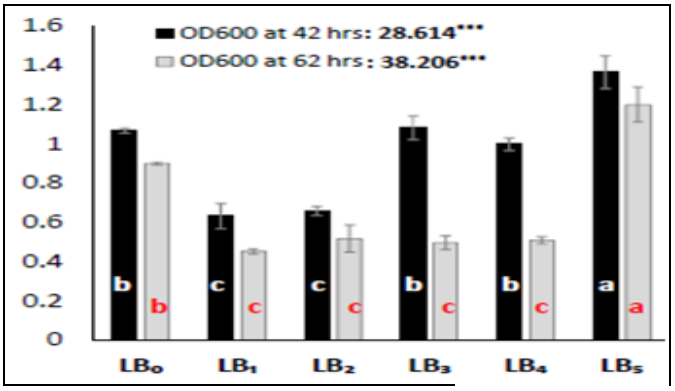

FIG. 1: CELL GROWTH RATE OF *B. SUBTILIS* LC4 AFTER 42 h AND 62 h CULTURES DEVELOPED WITH A SERIES OF COMBINATIONS OF LB LIQUID WITH EXTRACTS OF PEELS AND PEELED OFF POTATOES (AS SHOWN IN TABLE 1)

The *Bacillus subtilis* produces a large number of heterologous proteins in its growth medium [44]. This production rate and type of proteins vary from time to time that depends on the stage of typical growth phase. In this study, maximum extracellular proteins are estimated at 42 h of culture, while reduced at 62 h of cultures **Fig. 2**. Time interval from 42 to 62 h is major factor of the cell multiplication level as well as level extracellular productions in the medium, which is due to change in medium composition in corresponds to the usable level of nutrition of the medium. This nutritional stressed response could manifest a specific change in the metabolic activity of the cell to repress the biosynthesis of most normal cellular proteins under normal culture conditions, while induces a specific group of protein's synthesis to enable the survival of a cell under new conditions [45]. This tact of the microbial cells have attracted the industrial interest in microbial usage inspite of

toxic chemical products [46]. Among the cultures not only protein contents, alteration in the reducing sugars are observed from higher to lower in the harvests taken at 42 h and 62 h **Fig. 2**. Among the higher plants the level of cellular reducing sugars increases in the higher plants even animal cells under stressed conditions [47, 48]. Specific changes in the accumulation of certain metabolites under changes nutritional conditions increases the chances of survival of living organisms. Similarly, bacteria have adopted special mechanisms in the form accumulation of specific metabolites including specific proteins, free amino acids as well as reducing sugars under unfavourable growth limiting stressed conditions. In our experiment, production of reducing sugars and protein contents were higher in 62 h and also in 42 h culture which is uplifted state than to normal culture conditions [49]. This all is due to stress responses in the form of metabolic alterations, which are directed through specific signal transduction after sensing the applied stress including pH, temperature, salts and other oxidative stresses [50, 51].

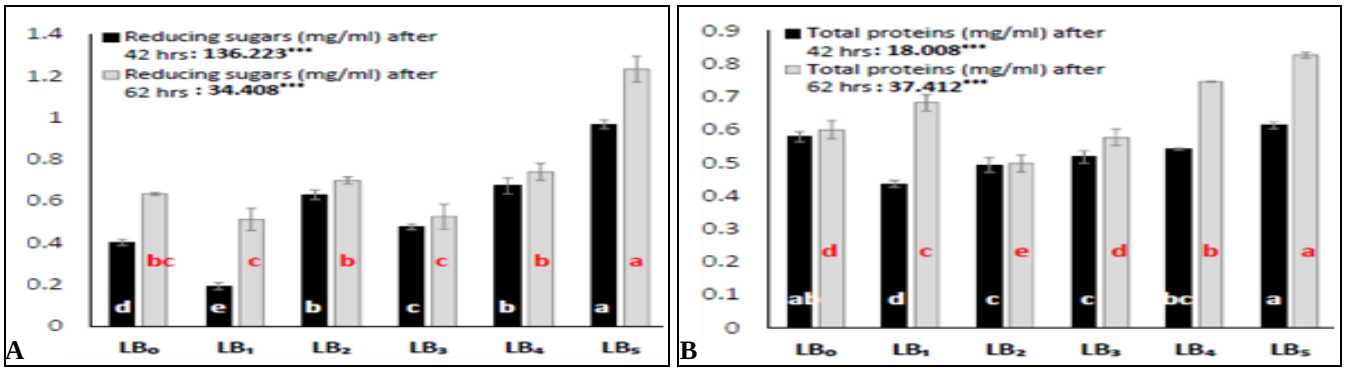

FIG. 2: DIFFERENTIAL LEVELS OF THE BIOSYNTHESIS OF EXTRA-CELLULAR PROTEINS (a) AND REDUCING SUGARS AFTER 42 h AND 62 h AMONG THE GROWTH CULTURES OF *B. SUBTILIS* LC4 CULTURES DEVELOPED WITH A SERIES OF COMBINATIONS OF LB LIQUID WITH EXTRACTS OF PEELS AND PEELED OFF POTATOES (AS SHOWN IN TABLE 1)

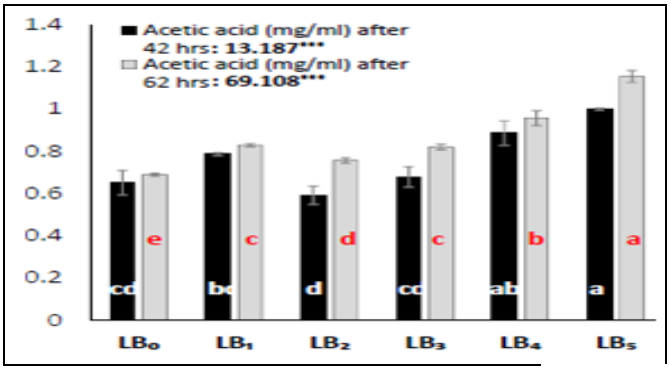

FIG. 3: PRODUCTION OF ACETIC ACID IN A SERIES OF CULTURES OF *B. SUBTILIS* LC4 AFTER 42 h AND 62 h. BACTERIAL NUTRIENT CULTURES DEVELOPED WITH DIFFERENT COMBINATIONS OF LB LIQUID WITH EXTRACTS OF PEELS AND PEELED OFF POTATOES (AS SHOWN IN TABLE 1)

Under above mentioned circumstances, production of acetic acid is observed among all cultures harvested at 42 as well as 62 h of incubation **Fig. 3**. Its biosynthesis is observed variable ($p \leq 0.05$) from culture to culture after 42 h, while trend of in both harvests is increasing similarly in each culture. The rotten potato's extract may contain suitable amounts of various sucrose contained substances in particular for *B. subtilis* cell multiplication. At initial stage of culture, it consumes the available carbon source for its rapid growth. With reference to previous reports for acetic production is reached to maximum concentration around 50 h of fermentation [52], while decreases after this time of incubation of *Acetobacter aceti* grown on sugarcane husk. In case of *B. subtilis*, production acetic acid is observed with increasing form even at 62 h of incubation. This increase or decrease in concentration of acetic acid depends on the presence or absence of oxygen, which induces oxidation of acetic acid. In our study, acetic acid production is higher in the harvested culture at 62 h, while at this stage cell growth rate is observed in decreasing trend.

Inspite of that the production of acetic acid in *B. subtilis* LC4 culture could be involved in shocking on a specific population of cell for sporulation induction [53]. The bacterial cells are used to convert itself from vegetative meristematic form to inactive spore forms under growth inhibiting stress conditions. A large number of biological processes are inhibited with the accumulation of acetic acid in cell cultures including the bioethanol production lignocellulose containing agricultural waste materials [54, 55]. While old reports suggest that at initial stages of sporulation, disappearance of acetic acid has observed. It could be major energy source of the stressed cells for their conversion to sporulation stage [56]. The utilization of acetates is inhibited might be due to the pH of medium. Further biotechnological improvement in the *Bacillus species* are required to search out acetate resistant strains or to induce resistance through genetic modifications.

CONCLUSION: Microbial growth and acetic acid production are focused on potential of rotten potatoes used as basal nutrient substrate. The *B. subtilis* LC4 showed high growth rate on peeled off extract of potato and also not bad on peels extract of potato cultures. It was reduced at 62 h harvest of culture incubation than 42 h on both potato's extracts also similar to standard TY-medium. Deficiency of nutrients lead to decrease in growth rate. At the same time production of acetic acid in all nutrient culture is observed under high level of reducing sugars. Both reducing sugars and acetic acid causes sporogenesis in certain population of cells in the culture. Peel and peeled off starch extract base rotten potato cultures have performed best for cell multiplication rate and biosynthesis of reducing sugars as well as acetic acid production. The rotten potato could be an important cheapest industrial substrate for the production of many more useful health promoting metabolites including acetic acid. As it is being produced and consumed at the range in millions of tons year^{-1}.

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CONFLICT OF INTEREST: No conflict of interest.

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