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EFFECTS OF ENVIRONMENTAL AND GROWTH CONDITIONS ON PROTEIN PRODUCED BY *Bacillus subtilis* ATCC21332 IN THE PRESENCE OF *Cymbopogon flexuosus* ESSENTIAL OIL

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ABSTRACT

The presence of antimicrobial substances at low concentration may act as chemical signals in altering metabolic processes that take place in bacteria. The chemical signals may activate lots of signal transduction that result in the secretion of bioactive proteins by the stimulated bacterial cells. The bioactive proteins production is strongly influenced by certain factors such as pH and nutrient sources. Therefore, the study of environmental stress factors and growth conditions is very important in inducing the production of bioactive proteins by microbes. The main objective of this study is to evaluate the effects of environmental stress factors and growth conditions on the production of bioactive proteins by *Bacillus subtilis* ATCC21332 in the presence of *Cymbopogon flexuosus* essential oil. The microbial proteins were produced by fermentation process using Nutrient broth with adjustment of pH media (pH 6, 7 and 8), and supplementation with 1% (w/w) of three different nutrients, including carbon sources (glucose, sucrose and starch), nitrogen sources (urea, casein and gelatin) and inorganic salts (calcium chloride, sodium nitrate, sodium dihydrogen phosphate) separately. The extracellular protein produced by *B. subtilis* ATCC21332 was isolated and analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to detect appearance of new protein bands. Results showed that new extracellular proteins were synthesized by *B. subtilis* ATCC21332 after being induced with *C. flexuosus* essential oil and grown in each media with 1% (w/w) of starch, 1% (w/w) of gelatin, 1% (w/w) of casein, 1% (w/w) of sodium dihydrogen phosphate and 1% (w/w) of calcium chloride with approximate size of 30.43 kDa, 30.66 kDa, 31.92 kDa, 31.35 kDa and 30.80 kDa respectively. Hence, *B. subtilis* ATCC21332 in the presence of 0.01 MIC *C. flexuosus* essential oil under different conditions are able to produce or secrete new extracellular proteins.

Key words: Protein, environmental and growth condition, *Bacillus subtilis* ATCC21332, *cymbopogon flexuosus*, essential oil

INTRODUCTION

Bacteria are exposed to constant variations of environmental stress in their growth conditions. Consequently, they may launch stress responses under stressful environments, including changes in temperature, pH, osmolarity, radiations as well as the concentration of nutritional factor and toxins production in order for them to adapt and survive. The stress responses consist of changes in characteristic that are related to the pattern of gene expression. This stress response will help the bacteria to protect vital processes and to restore homeostasis, and increase cellular resistance against the environmental stresses (Aertsen & Michiels, 2004). Antimicrobial agents represent one of the stresses that could affect the bacterial growth. The antimicrobial agents or antibiotics with different structures and modes of action at low concentration or sub-Minimal Inhibitory Concentration (sub-MIC) are able to cause big changes in gene transcription process (Davies *et al*., 2006). In this stressful condition, the bacterial protein level production was higher than normal condition in order to survive in those insults (Tanaka *et al*., 2005).

Essential oils (EOs) extracted from plants are one of natural products that can be used as antimicrobial agents. They have been used traditionally for various applications in food as well

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as commercial application in health-related areas (Vimalanathan & Hudson, 2014). *Cymbopogon* species (Poaceae), which mostly distributed in Asia and in Africa, are one of aromatic and medicinal plants that produce essential oils with commercial interest. Lemongrass oil, a main source of natural citral is commonly obtained from *C. flexuosus* (Nees ex Steud) and mainly produced in India. *C. flexuosus* essential oils mainly composed of geranial and neral with total content of citral (63.3%) (Surburg & Panten, 2006). A study by Vora *et al.* (2014) reported that *C. flexuosus* is effective against *A. aegypti*. Besides, protein secreted by *L. plantarum* ATCC8014 after inducing with *C. flexuosus* exhibited bactericidal effect towards *K. pneumonia* and *S. typhimurium* (Hanina *et al*., 2015).

Members of the genus *Bacillus* are known producers of a wide spectrum of bioactive peptides which have a great potential for biotechnological and biopharmaceutical applications. Some of the compounds are ribosomal origin, while the others belong to surfactic, utirin and fengcin families, which are formed by nonribosomal peptide synthetases. Due to this chemical and physical diversity of peptide antibiotics, *Bacillus* sp. are potentially useful for therapeutic application as well as agri-food industry (Vijayalakshmi & Rajakumar, 2010; Leclere *et al*., 2005). Besides, *Bacillus subtilis* is able to synthesize antibiotic and extracellular enzymes at the end of its exponential growth as well as during encountering nutrient limitation. The major extracellular proleolytic enzymes include neutral protease, subtilisin or alkaline (Piggot, 2009; Sloma *et al*., 1990). Previous study reported that *B. subtilis* ATCC21332 in the presence of *C. flexuosus* essential oil are able to produce new protein recognized as Bacillopeptidase F (Hanina *et al*., 2014).

Optimization of fermentation process is very important to improve the protein production. In order to produce maximum yield of protein, bacteria are grown in fermenters under optimum conditions (Pant *et al.*, 2015). In the past, the optimization of protein production by bacterial strains was performed by empirical approaches such as studying the effects of medium compositions on protein yields (Westers *et al*., 2004). The proteins production is also strongly influenced by many other environmental factors including pH, temperature, incubation period, cell density and nutrient sources (Vijayalakshmi & Rajakumar, 2010). However, most reported findings, which are based on conventional study using one variation of parameter, resulted in only an 'apparent' set of optimal conditions. Therefore, understanding and modelling of both conventional and interactive effects of important parameters are essential to obtain a high-performance synthesis (Châabouni *et al.*, 2006).

It is well studied that bacteria are exposed and respond to many extracellular signals in the environment. However, there are limited studies on natural compound used at low concentration act as chemical signal to activate biological function in bacteria. The principle behind this study is the natural plant antimicrobial, *C. flexuosus* essential oil at 0.01 MIC has the ability to give global changes in gene transcription process by activating gene expression in producing new protein. The protein produced by the bacteria in the presence of *C. flexuosus* essential oil could be enhanced by optimizing the bacterial growth conditions. Therefore, this study aimed to explore the effect of *C. flexuosus* oil as bacterial stress factor in stimulating bioactive protein produced by *B. subtilis* ATCC21332 cultured in different growth condition.

MATERIALS AND METHODS

Essential Oils, Bacterial Strain and Culture Conditions

Cymbopogon flexuosus essential oil was obtained from Al-Muqarram Holdings Sdn Bhd. *Bacillus subtilis* strain ATCC21332 was purchased from American Type Culture Collection (ATCC), and was grown in Nutrient Broth (NB, Merck, Germany) and maintained on Nutrient Agar (NA, Merck, Germany).

Optimization of Microbial Protein Production via One-Parameter-at-a-Time Approach

The optimization of protein production was done by varying one-parameter-at-a-time approach, based on method by Vijayaraghavan *et al*. (2014). The pH of media (pH 6, 7 and 8) and 1% (w/w) of medium components such as carbon sources (glucose, sucrose and starch), nitrogen sources (urea, casein and gelatin) and inorganic salts (calcium chloride, sodium nitrate, sodium dihydrogen phosphate) were optimized. The physical factors, including fermentation period (72 h), temperature (30°C), bacterial densities (8 h) were evaluated before proceeding with the optimization of protein production.

Fermentation Process of Microbial Protein

Fermentation process was done according to method by Hanina *et al*. (2014). A 10 µl of an overnight grown of *Bacillus subtilis* was cultured into 50 ml of NB before further shaken vigorously at 30°C. After reaching log phase of 8 h of cultivation, the bacterial cultures were added with 0.01 MIC of *C. flexuosus*, before being further fermented in certain media either with three different pH (6, 7 and 8) or with three different sources of

nutrients (carbon, nitrogen, inorganic salts) separately. The cultures were further shaken vigorously at 30°C for 72 h. A culture with addition of inducer and normal pH of NB without the addition of nutrients was served as control (C).

Microbial Protein Extraction, Isolation and Identification

The secreted extracellular protein was extracted and isolated based on method by Bajpai *et al*. (2016). The bacterial cells were separated by centrifugation (Hanil, Combi 514R, Korea) at 12,000 ×g for 15 min at 4°C, and filter sterilized through a 0.2 µm syringe filter. The supernatant was then precipitated with 80% (w/v) of ammonium sulphate (R&M Chemicals, Malaysia) before being kept for 1 h at 4°C. The precipitated proteins were collected by centrifugation at 15,000 ×g for 20 min at 4°C. The resulting pellets were resuspended in phosphate-buffered saline (PBS, pH 7.4). The protein suspensions were desalted using dialysis tubing (Sigma-Aldrich, USA) with 12400 Da cutoff for 24 h at 4°C, before being further evaluated for protein analysis using sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (SDS-PAGE). The dialyzed proteins were then mixed with Laemmli buffer (Bio-Rad, Singapore) in 1:1 ratio and heated at 95°C for 5 min before being loaded into SDS-PAGE gel.

Protein Identification using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The dialyzed proteins were analyzed for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on Any kD™ Mini-PROTEAN® TGX™ Precast Gel in a Protean III electrophoresis system (Bio-Rad, USA) with Precision Plus Protein™ Dual Xtra Standards (10-250 kDA) (Bio-Rad, USA) based on method by Laemmli (1970). The protein bands were visualized by staining with Biosafe coomasive blue (Bio-Rad, USA).

RESULTS AND DISCUSSION

B. subtilis is known as a Gram-positive bacterium which lacks of an outer membrane. Thus, *B. subtilis* are able to secrete large amount of extracellular proteins directly into its environment and simplify the downstream processing of the purified secreted proteins. This bacterium is regarded as a cell factory in industries of enzymes and biopharmaceutical (Himanen *et al.,* 1990; Schallmey *et al.,* 2004; Westers *et al.,* 2004). It has been reported that *B. subtilis* ATCC21332 in the presence of *C. flexuosus* essential oil are able to produce a new protein with 30 kDa in size which is recognized as Bacillopeptidase F (Hanina *et al*., 2014). This present study focused on the optimization of Bacillopeptidase F production as well as determination of new extracellular proteins secreted by *B. subtilis* ATCC21332 in the presence of *C. flexuosus* essential oil under different growth conditions. The bacterial cells were cultivated in media with 0.01 MIC of *C. flexuosus* essential oil as stress inducer and grown under different pH and nutritional factors. The extracellular proteins secreted were extracted before being further identified using SDS-PAGE analysis.

Effects of pH on Microbial Protein Production

The SDS-PAGE analysis indicated a 30 kDa protein secreted by *B. subtilis* ATCC21332 which are cultured in the presence of *C. flexuosus* essential oil and grown under different pH media of 6, 7 and 8 or without pH adjustment as shown in Fig. 1. The intensity of protein bands produced by stress induced *B. subtilis* ATCC21332 grown in media with pH 6 and 7 were highly expressed compare to media with pH 8. However, there was no any new protein band produced by *B. subtilis* ATCC21332 induced with *C. flexuosus* essential oil and cultivated in media with three different pH. This may occur due to the bacterial itself in which can still stabilize its cytoplasmic pH even though being exposed with various range of extracellular pH in the environment.

B. subtilis are regarded as neutralophilic bacteria since they are able to maintain their cytoplasmic pH within a narrow range of pH, although being exposed to wide range of extracellular pH (Martinez *et al.*, 2012). It has been acknowledged that the external pH will partly determine the cytoplasmic or intracellular pH, in which may affect protein stability, enzyme activity and reactions rates, structure of nucleic acids and other biological molecules (Slonczewski *et al.,* 2009).

Effects of Carbon Sources on Microbial Protein Production

Carbon sources are known as an essential element for bacterial growth and metabolism of bacteria (Khusro *et al.,* 2016). These carbon sources usually degraded at cellular level as monosaccharides, disaccharides before further transported in the cells and metabolized by the bacteria. *Bacillus* sp. are able to degrade polysaccharides such as starch using extracellular enzymes into monosaccharide and disaccharides before being further transported and utilized by cells. The disaccharides such as sucrose or degradation of starch to maltose by microbes are then hydrolyzed to monosaccharides inside the cells by specific

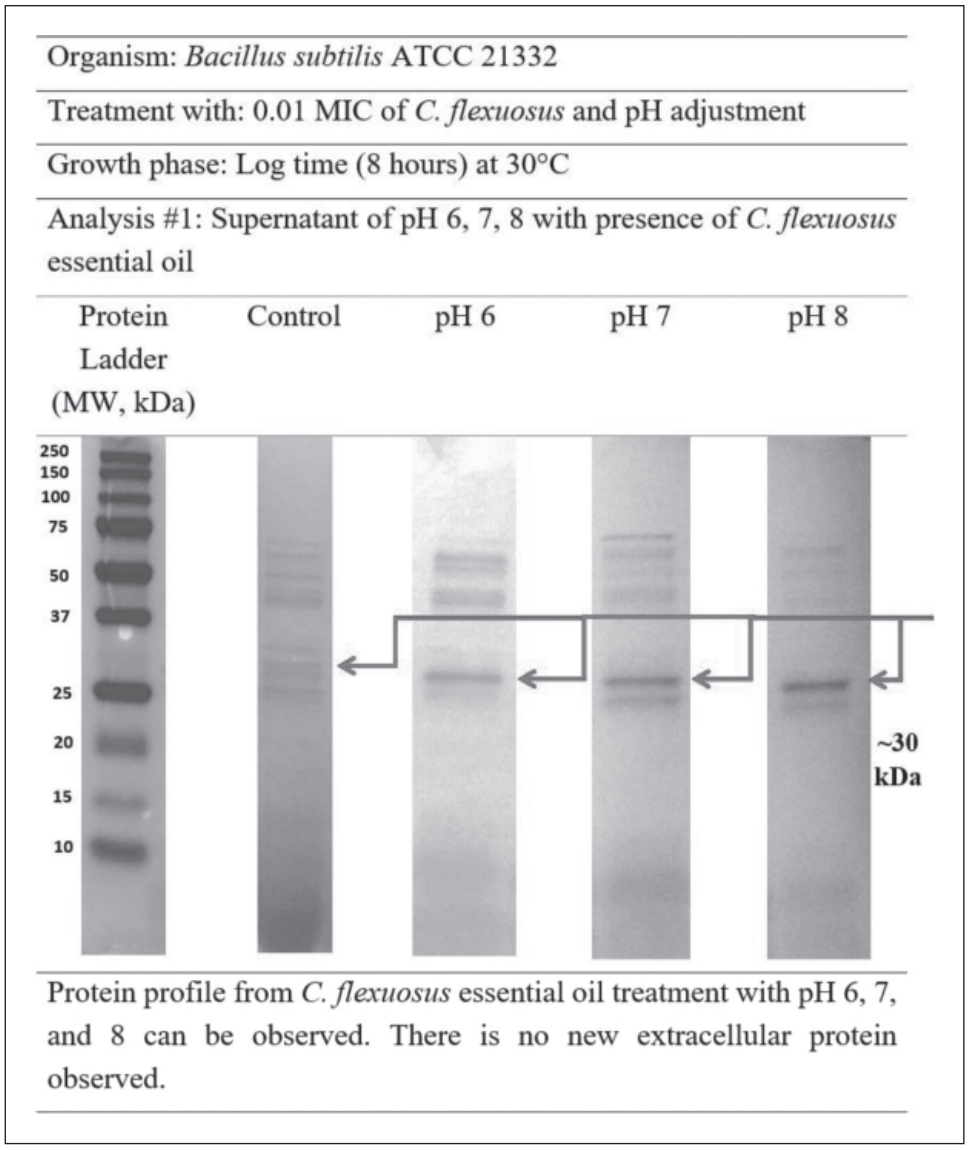

Fig. 1. SDS-PAGE analysis on extracellular proteins produced by *B. subtilis* ATCC21332 in the presence of *C. flexuosus* essential oil with media pH adjustment at 30°C for 72 h of fermentation. Note: Lane (1) Protein Ladder (10-250 kDa); Lane (2) in the presence of *C. flexuosus* only; Lane (3) protein with pH 6 and in the presence of *C. flexuosus*; Lane (4) protein with pH 7 and in the presence of *C. flexuosus*; Lane (5) protein with pH 8 and in the presence of *C. flexuosus.*

enzymes. For example, sucrose is degraded to glucose by the enzyme sucrase, and maltose is digested to glucose by the enzyme maltase (Ray & Bhunia, 2007).

The natural habitat of *B. subtilis,* which is soil contains various carbohydrates such as polysaccharides that can be derived from plants, animals and microbes. Therefore, lots of polysaccharides degrading enzymes are produced by *B. subtilis* including α-amylase, pullulanase, endo-β-1,4-mannase, levanase and many more. These enzymes are secreted into its environment for breaking down the polysaccharides into smaller entities before being further taken up and metabolized by bacterial cells (Deutscher *et al.,* 2002).

In this study, a 30 kDa protein band which is recognized as Bacillopeptidase F was produced by *B. subtilis* ATCC21332 in the presence of *C. flexuosus* essential oil and cultivated in each media with 1% (w/w) of sucrose, starch and glucose as carbon source as shown in Fig. 2. Nevertheless, the intensity of 30 kDa protein bands produced by stress induced *B. subtilis* ATCC21332 cultured in media with 1% (w/w) of starch was highly expressed compared to media with the supplementation of glucose and sucrose or without the addition of any carbon source.

As shown in Fig. 2, there is a new extracellular protein with approximate size of 30.43 kDa was synthesized by *B. subtilis* ATCC21332 after being

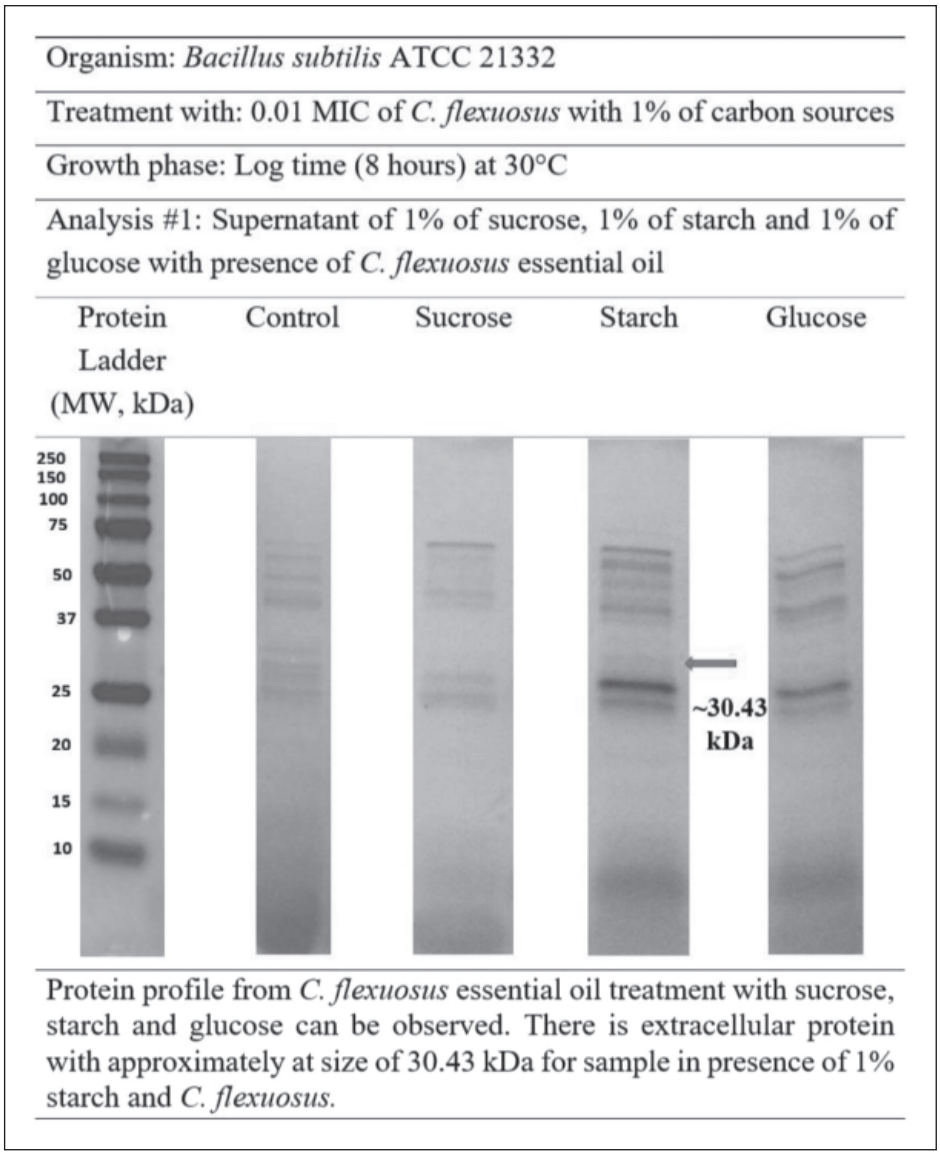

Fig. 2. SDS-PAGE analysis on extracellular proteins produced by *B. subtilis* ATCC21332 in the presence of *C. flexuosus* essential oil with 1% of carbon sources at 30°C for 72 h of fermentation. Note: Lane (1) Protein Ladder (10-250 kDa); Lane (2) in the presence of *C. flexuosus* only; Lane (3) protein with 1% of sucrose and in the presence of *C. flexuosus*; Lane (4) protein with 1% of starch and in the presence of *C. flexuosus*; Lane (5) protein with 1% of glucose and in the presence of *C. flexuosus.*

induced with *C. flexuosus* essential oil and grown in media with 1% of starch during 72 h of fermentation period at 30°C. This showed that *B. subtilis* ATCC21332 are able to utilize 1% (w/w) of carbon sources under mild stress condition in the presence of *C. flexuosus* essential oil.

Effects of Nitrogen Sources on Microbial Protein Production

Nowadays, there are a number of studies on the influence or effect of nitrogen source in promoting bacterial growth (Venil & Perumalsamy, 2009). One of the important reasons is that nitrogen gas is commonly known and easily available in surrounding environment. Thus, it may affect the growth system of microorganisms either in high or low concentration. The presence of Nitrogen dioxide (NO_2) and Nitric oxide (NO) in surrounding air usually give a positive impact in enhancing the growth of some important bacteria such as *Micrococcus luteus*, *Micrococcus roseus*, *Serratia marcescens*, *Bacillus subtilis* and many others (Vora *et al*., 2014). Even though there are some probabilities that nitrogen sources will provide a toxicity and harmful effect to the bacteria, but this may only occur in high amount or concentration of nitrogen when the presence of acids is high. It has been reported that there is no bactericidal effect on

selected bacteria when they were exposed to certain amount or concentration of NO and NO_2 in their growth media (Santos *et al*., 2002).

In this present study, two new extracellular proteins with approximate size of 30.66 kDa and 31.92 kDa were secreted by *B. subtilis* ATCC21332 after being induced with *C. flexuosus* essential oil and grown in each media with 1% (w/w) of gelatin and 1% (w/w) of casein subsequently as shown in Fig. 3. Besides, the intensity of 30 kDa protein bands produced by stress induced *B. subtilis* ATCC21332 cultured in media with 1% (w/w) of urea was highly expressed compared to media with the supplementation of the other two nitrogen sources (gelatin and casein) or without the addition of any nitrogen source. These may due to response of cells towards changes in the environments. Furthermore, the resulting over- or under-expressed proteins may directly related to a given exogenous stimulus, which regarded in the precise regulation of cellular activities (Hu *et al*., 2003).

Effects of Inorganic Salts on Microbial Protein Production

The bacterial growth and protein production are affected by the different concentrations of inorganic salts presence in growth media (Yamazaki *et al*., 2006). Some bacterial species may require inorganic

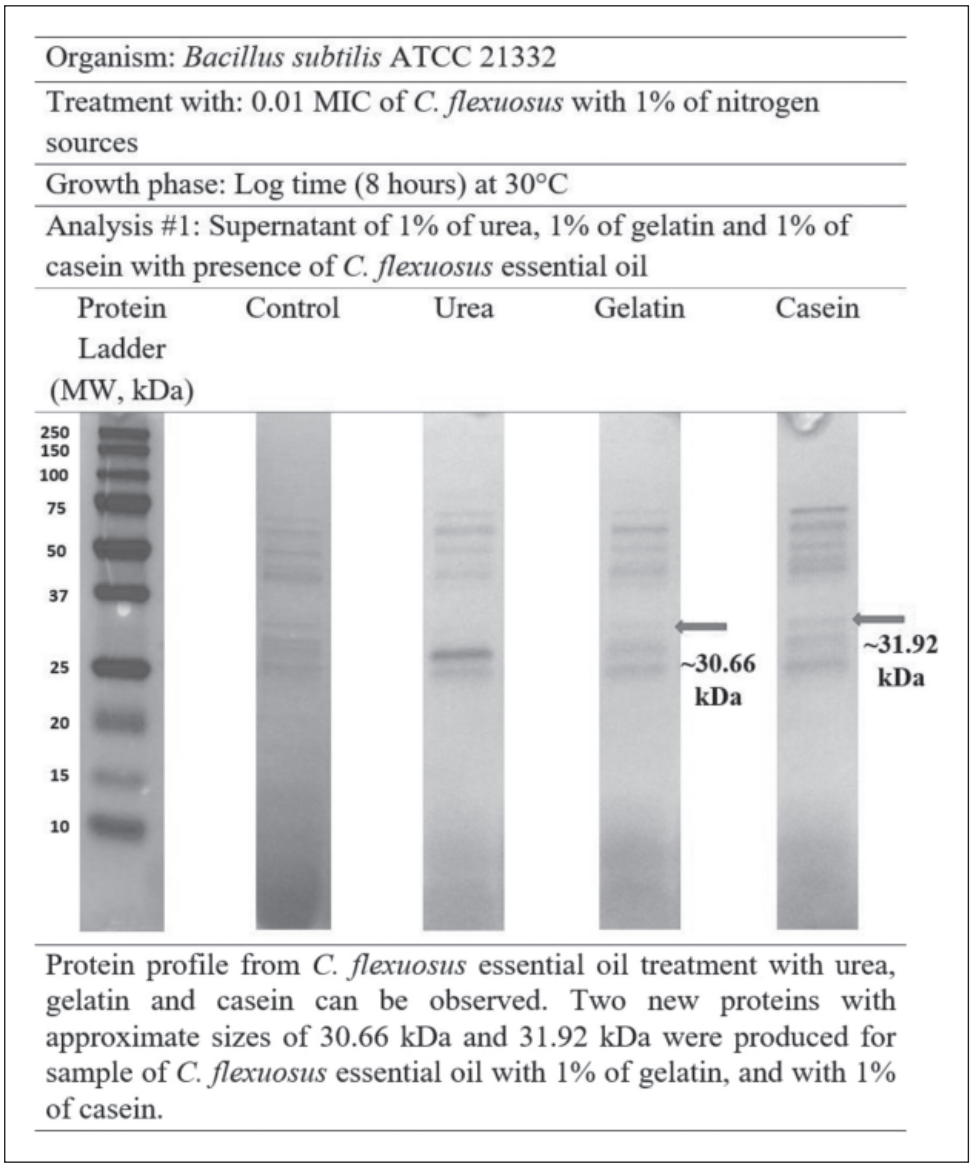

Fig. 3. SDS-PAGE analysis on extracellular proteins produced by *B. subtilis* ATCC21332 in the presence of *C. flexuosus* essential oil with 1% of nitrogen sources at 30°C for 72 h of fermentation. Note: Lane (1) Protein Ladder (10-250 kDa); Lane (2) in the presence of *C. flexuosus* only; Lane (3) in the presence of 1% urea and *C. flexuosus*; Lane (4) in the presence of 1% gelatin and *C. flexuosus*; Lane (5) in the presence of 1% casein and *C. flexuosus*.

salts in order to carry out their cellular activities. Although some of them can grow without the presence of inorganic salts, but they may also tolerate towards small amounts of inorganic salts in their growth media. For example, *Vibrio* sp., in which has an internal osmolyte concentration require inorganic salts from extracellular environment in order to maintain osmotic balance over the cell membrane. However, *E. coli* showed a different patent of growth in which they may have optimal growth in the absence of inorganic salts and attenuated in the presence of inorganic salts (Abdulkarim *et al*., 2009). However, it has been reported that inorganic salts can increase the production of bacteriocin by *Lactobacillus* sp. (Neysens *et al*., 2003).

In this study, new extracellular proteins with approximate size of 31.35 kDa and 30.80 kDa were synthesized by *B. subtilis* ATCC21332 in the presence of *C. flexuosus* essential oil and cultured in each media with 1% (w/w) of sodium dihydrogen phosphate and 1% (w/w) of calcium chloride respectively as shown in Fig. 4. Moreover, the intensity of 30 kDa protein bands produced by stress induced *B. subtilis* ATCC21332 cultivated in media with 1% (w/w) of calcium chloride was highly

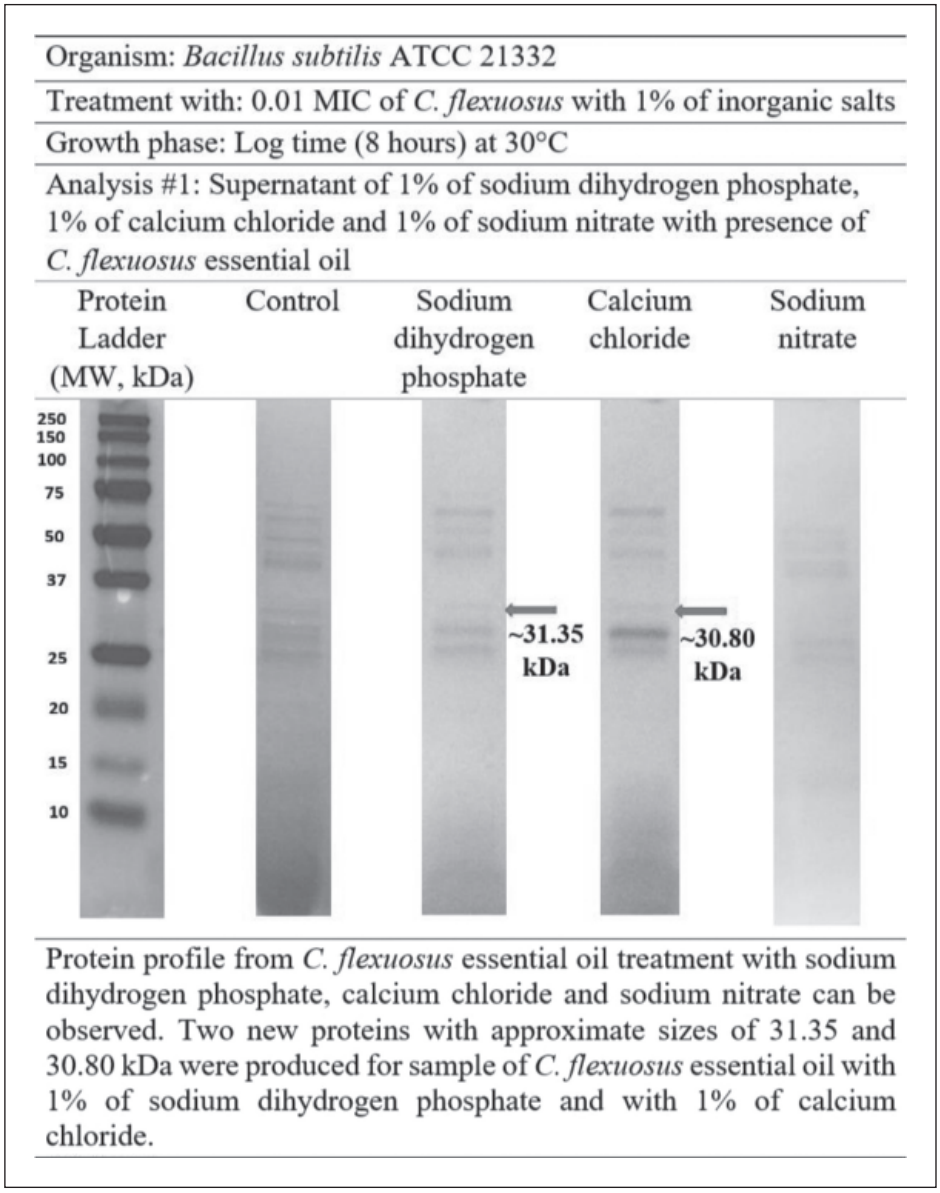

Fig. 4. SDS-PAGE analysis on extracellular proteins produced by *B. subtilis* ATCC21332 in the presence of *C. flexuosus* essential oil with 1% of inorganic salts at 30°C for 72 h of fermentation. Note: Lane (1) Protein Ladder (10-250 kDa); Lane (2) in the presence of *C. flexuosus* only; Lane (3) in the presence of 1% sodium dihydrogen phosphate and *C. flexuosus*; Lane (4) in the presence of 1% calcium chloride and *C. flexuosus*; Lane (5) in the presence of 1% sodium nitrate and *C. flexuosus*.

expressed compared to media with the supplementation of the other two inorganic salts or without the addition of any inorganic salts. This may occur due to osmoregulation by bacterial cell in order to maintain osmotic balance and conduct some cellular activities for their growth.

CONCLUSION

B. subtilis ATCC21332 in the presence of *C. flexuosus* essential oil as stress inducer and under different growth conditions are able to optimize the Bacillopeptidase F production as well as secreted new extracellular proteins. Further identification of protein produced should be carried out using mass spectrometry analysis in future study.

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