The characteristics of isolate f

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A bacterium labeled as isolate F that exhibited keratinolytic activity from the Biomass Laboratory UPM was selected for the study of effect of different nitrogen sources on keratinase production. The physical and biochemical characteristics of the isolate F was studied before starting the optimization of medium with different nitrogen sources. Gram staining method which involved 4 different types of reagents such as crystal violet, gram's iodine, ethyl alcohol 95% and safranin was used to identify isolate F. Through this method, isolate F was identified as Gram-negative bacterium since it was observed to be pink colour when viewing under microscope. This indicated that the isolate F has outer membrane with higher lipid content which prevents the crystal violet stain from reaching the peptidoglycan layer in the periplasm. Hence, the alcohol will wash away the crystal violet stain in the outer membrane of the bacterium and safranin will stain the cell wall of isolate F in pink colour. Besides, the morphology of the isolate F could be observed as thin, short-rod shaped bacteria which occurred singly. Based on those characteristics stated previously, we could suspect that the isolate F would be a member of Bacillus genus, Chryoseobacterium genus, Xanthomonas genus or Vibrio genus. From this piece of information, isolate F probably is serine type protease which can function optimally under alkaline condition. The growth profile of isolate F was constructed in order to determine the suitable incubation time for inoculums preparation. Spectrophotometer measurement at 600 nm was used to reflect the cell growth. As shown in Figure 4. 1, isolate F was observed to display a typical four phase pattern of growth which included lag phase, log phase, stationary phase and death phase. Log phase for isolate F occurred between 0 hour to 4

hours where the bacterium was still adapting to the new environment in the fresh medium and experienced slow increased in cell numbers. After 4 hours of incubation, the isolate F culture entered log phase during which the optical density and turbidity of the cell culture was noticed to be increased double with cultivation time. This indicated their population was doubled and the growth rate of the cells was directly proportional to the period of fermentation. The maximal growth rate of isolate F was attained at 12 hours of incubation. Stationary phase occurred between 12 to 14 hours where the rate of multiplication equals the rate of death due to nutrients became limiting factor or accumulation of inhibitory end products. The death phase of isolate F could be seen after 14 hours of incubation as the condition in the medium was unable to support the growth of the bacterium. Hence, an incubation time of 10 to 12 hours was sufficient for preparing inoculum for the fermentation process. Figure 4. 1 Growth profile of isolate F. Error bars (±S. D.) are shown when larger than the symbolNitrogen is major nutrient with lots of useful peptides and plays a very significant role especially in medium preparation. It had been shown that the different nitrogen sources could affect cell metabolism in various pathway. In order to quantify these affect, different nitrogen sources were supplemented into flask containing basal medium and chicken feather to access their suitability on growth and keratinase production of local isolate F. Typically, nitrogen sources are classified as either organic or inorganic. Organic nitrogen source is more complex and contain components which are difficult to be estimated. Generally, this form of nitrogen is rich in amino acids, vitamins and growth factors. On the other hand, inorganic nitrogen source is simpler form and

contains defined components in measured amount. In this study, sodium nitrate is an example of inorganic nitrogen source while yeast extract, peptone, tryptone and soyabean cake are examples of organic nitrogen sources. All the nitrogen sources were fixed at 0. 01 g/ L or 10 % (w/v) nitrogen content to ensure same starting nitrogen available for fermentation of local isolate F. Effect of different nitrogen sources on local isolate F was determined by monitoring optical density, colony forming unit (cfu/ ml) as well as enzyme activity and soluble protein. Table 4. 1 Optical density profile of respective nitrogen sources by local isolate FNitrogen sourcesOptical density at 600 nm0 hour24 hours48 hours72 hours96 hours120 hoursControl (chicken feather)0. 107 (±0. 005)0. 234 (±0. 014)0. 300 (±0. 010)0. 529 (±0. 008)0. 482 (±0. 014)0. 411 (±0. 006)Yeast extract0. 148 (±0. 027)0. 208 (±0. 023)0. 369 (±0. 007)0. 567 (±0. 016)0. 538 (±0. 014)0. 481 (±0. 033)Peptone0. 161 (±0. 161)0. 295 (±0. 025)0. 374 (±0. 004)0. 601 (±0. 014)0. 552 (±0. 013)0. 607 (±0. 008)Tryptone0. 174(±0. 027)0. 315 (±0. 012)0. 355 (±0. 029)0. 636 (±0. 018)0. 587 (±0. 054)0. 558 (±0. 038)Soyabean cake0. 125 (±0. 008)0. 250 (±0. 007)0. 277 (±0. 011)0. 443 (±0. 020)0. 369 (±0. 018)0. 366 (±0. 037)Sodium nitrate0. 105 (±0. 005)0. 231 (±0. 008)0. 286 (±0. 016)0. 416 (±0. 008)0. 365 (±0. 026)0. 325 (±0. 025)*Values are expressed as mean ± standard deviation of three independent experimentsThe optical density was used to reflect the cell concentration. Based on Table 4. 1, it could be observed that all the medium with or without selected nitrogen source exhibited a gradual increasing trend with the highest optical density at 72 hours of incubation and decreased slowly after 84 hours of incubation. At 0 hour of fermentation, the initial

starting optical density or cell concentration was maintained at the range of 0. 100 to 0. 200 for all the nitrogen sources. The results of fermentation had also shown that the medium supplemented with tryptone showed the highest optical density of 0. 636 ± 0. 018 at 72 hours incubation, followed by medium supplemented with peptone (0. 601 ± 0. 014) and yeast extract (0. 567 ± 0. 016), in contrast to that of control medium with an absorbance of 0. 529 ± 0 008. Meanwhile, the medium with soyabean cake and sodium nitrate as nitrogen sources obviously depicted a decreased in absorbance value of 0. 443 ± 0. 020 and 0. 416 ± 0. 008 respectively when compared with that of the control. In a study by Jeong et al. (2010), Stenotrophomonas maltophilia R13 reported an approximately double increased in maximum optical density in the presence of polypeptone (0. 654 ± 0. 018), tryptone (0. 545 ± 0. 020), yeast extract (0. 512±0. 026) and sodium nitrate (0. 577 ± 0. 017) in comparison with control’s absorbance value of 0. 285±0. 045. These reported results was in accordance to the previously stated optical density profile for local isolate F for most of the nitrogen sources except the sodium nitrate which was inorganic nitrogen source. One of the reasons might be due to the different species or origin of bacterium exhibit differential preferences toward either organic or inorganic nitrogen sources added.

Figure 4. 2 Logarithms of colony forming unit profile for respective nitrogen sources by local isolate F. Symbols: diamond, control (chicken feather only); rectangle, yeast extract; triangle, peptone; cross, tryptone; star, soyabean cake; circle, sodium nitrate. Each data was the mean of duplicate experimentsThe cell biomass of isolate F could also be illustrated by using colony forming unit (cfu/ mL). As shown in Figure 4. 2, the graph of

logarithms of colony forming unit for all tested nitrogen sources reflected sigmoid shaped curve. Logarithms of colony forming unit profile and optical density profile for isolate F had coincidence trends in terms of resultant effect of nitrogen source with noticeable maximal cell growth at the third days of incubation. The most encouraging growth yield was shown by the basal media with tryptone as nitrogen source as well where it grew from initial cell density of 1. 39 × 109 cfu/ mL to a cell density of 29. 9 × 109 cfu/ mL within 3 days of cultivation. Basal medium supplemented with peptone and yeast extract also showed positive effect in term of maximal growth yield with 27. 5 × 109 cfu/ mL and 26. 9 × 109 cfu/ mL bacterial cells respectively in comparison with control (16. 9 × 109 cfu/ mL). On the other hand, the repression in bacterial growth yield was observed in culture media supplemented with soyabean cake and especially for sodium nitrate which depicted the lowest yield of 14. 3 × 109 cfu/ mL. These findings were similar to those reported by Jeong et al. (2010), who stated that the cell growth of Xanthomonas sp. P5 was boosted up to 14. 1 × 108 cfu/ mL, 12. 8 × 108 cfu/ mL and 12. 0 × 108 cfu/ mL in the presence of organic nitrogen sources such as yeast extracts, tryptone and polypeptone respectively which were approximately 10 times higher growth rate than that of control. However, the reported cell density in reference journal seemed to be 10 times lower than that of the findings in this study. This might be due to a higher inoculum size had been used in this study in order to reduce the lag phase and achieve high viability in the starter culture. Figure 4. 3 Keratinase enzyme activity profile for respective nitrogen sources by local isolate F. Symbols: diamond, control (chicken feather only); rectangle, yeast extract;

triangle, peptone; cross, tryptone; star, soyabean cake; circle, sodium nitrate. Each data was the mean of three independent experiments. Error bars (±S. D.) were shown when larger than the symbol. Enzyme activity is the determinant factor in the selection of the nitrogen sources for optimal keratinase production by local isolate F. Synthesis of extracellular keratinase could be either constitutive or partially inducible. The understanding of cell growth associated with maximum enzyme production period could be very important for identifying the fermentation period for maximal enzyme productivity. In this case, the maximal enzyme productivity of isolate F was occurred at 72 hours of incubation. In this study, the highest enzyme production for isolate F had been achieved in the medium added with tryptone (26. 0 U/ mL) in which 52. 94 % increment was being observed. This was then followed up by peptone (23. 0 U/ mL), yeast extract (22. 0 U/ mL) which also showed enhancing effect on keratinase production. Some of the researchers had reported that the addition organic nitrogen source such as yeast extract, tryptone, and peptone improved keratinase production in many keratinolytic bacteria (Cai and Zheng, 2009; Mazatto et al., 2009; Anbu et al., 2006; Jeong et al., 2010). For instance, Kainoor and Naik (2010) on the study of Bacillus sp. JB 99 demonstrated a 4. 4 fold, 3. 4 fold and 2. 4 fold increment in keratinase activity upon addition of yeast extract, peptone and tryptone respectively. Besides, production of keratinase from Bacillus lichenformis PWD-1 also increased by 12 % when supplemented with tryptone as published by Cheng et al. (1995). However, it was not necessary applicable to all the organic nitrogen sources. It had been illustrated in Figure 4. 3 that soyabean cake achieved enzyme activity lower than that of

control. Evidence was provided by Bernal et al. (2003) which stated that addition of soyabean meal could lower the keratinase activity of Kucuria rosea by about 23 %. For inorganic nitrogen sources, they were frequently reported to reduce the microbial keratinase production (Tiwary and Gupta, 2010; Brandelli and Riffel, 2005; Park and Son, 2009; Saibabu et al., 2013). As mentioned by Ni et al. (2011), the keratinase activity decreased drastically by 65. 55% when cultivated with sodium nitrate. The earlier research were in accord with the present study on isolate F where the inorganic nitrogen source, sodium nitrate was found to have the lowest keratinase activity of 13. 0 U/ mL with a reduction of 23. 53 % in enzyme activity when compared to that of control and other nitrogen sources. However, sodium nitrate had also reported to enhance keratinase production of bacteria in some cases (Anbu et al., 2006; Kansoh et al., 2009). At this point, it was interesting to found out that the optical density, colony forming unit and enzyme activity profile for isolate F had comparable patterns and results for all tested nitrogen sources. In this present study, all three parameters proposed that 72 hours of cultivation was ideal for maximum growth rate and enzyme activity by local isolate F. This was predictable as an increase in bacterial cell density will generally have cumulative positive effect on keratinase enzyme activity under optimal condition. Similarly, Gupta and Ramnani (2006) as well as Kanchana (2012) also reported the highest keratinase activity in Bacillus licheniformis RG1 and Bacillus sp. respectively at 72 hours of growth. In addition, all three profiles also depicted a decreasing trend in growth and enzyme activity by strain F after 3 days of fermentation. It could be assumed that the loss of cell biomass and

enzyme activity probably due to end product feedback inhibition, nutrient depletion or enzymatic autolysis. Besides, stimulating effect of both cell growth and enzyme activity in isolate F were also seen for medium supplemented with tryptone, peptone and yeast extract respectively in decreasing order. These findings suggested synthesis of keratinase enzyme was inducible for most organic nitrogen sources. An overall look at the results revealed that the most favorable nitrogen source by isolate F was tryptone. This is because tryptone is complex nitrogen source which consists of enzymatic digest of casein. Casein is the main protein of milk rich in amino acid nitrogen which can promote the growth of bacterium. Peptone ranked secondly in term of preference of utilization by isolate F as nitrogen sources followed by yeast extract but was not significantly difference. Peptone is casein from meat or milk which is digested by pepsin while yeast extract contains hydrolysate of yeast cells, tris elements and vitamin B. Both nitrogen sources are also excellent natural sources of amino acids and peptides in sustaining the growth of bacterium. Although yeast extract was frequently reported by researchers as the most preferable organic nitrogen sources, however it does not tally with the present study (Saibubu et al., 2013; Kainoor and Naik, 2010; Jeong et al., 2010; Tiwary and Gupta, 2010). This might be due to influence of organic nitrogen sources on keratinase productions might be species-specific and differ among various bacteria. On the other hand, the induction of cell growth and keratinase activity were restricted in the presence of soyabean cake and sodium nitrate, suggesting that repression associated mechanism had happened. In other words, the biosynthetic pathway for the transcription and expression of keratinase gene

is interfered by their corepressors (Grazzotin et al., 2007). Soyabean cake used in this study was an organic biomass waste product obtained after soya sauce fermentation and had relatively high nitrogen content of about 17%. However, soyabean cake was unflavourable to provide nutrients for growth of isolate F since it showed repressive effect on keratinase production. This phenomenon might be caused by the insoluble properties of soyabean cake which eventually lead to lesser nutrients available for utilization by isolate F to boost up the enzyme productivity. In similar manner, addition of sodium nitrate also inhibited the keratinase activity because it is chemically synthesized, inorganic nitrogen source which lacks of growth factors and vitamin. Hence, its nutrient content is lesser than that of organic nitrogen source and does not exert much positive effect on keratinase production by isolate F. These findings suggested isolate F demonstrated a better performance for most organic nitrogen sources compared to inorganic nitrogen sources. Based on the results as well, it could be observed that control with no nitrogen source had relatively high enzyme activity and cell growth. This indicated that the isolate F possessed the ability to utilize the end products of its hydrolysed feather as its nitrogen sources. Thus, it could be deduced that chicken feathers as sole nitrogen were sufficient enough to support the growth and keratinase production by isolate F. This statement was supported by Sivakumar et al. (2013) on the study of Bacillus cereus TS1 and Matikevičienė et. al. (2011) on the study of Actinomyces fradiae 119.

Table 4. 2 Soluble protein concentration profile based on Lowry method at 750 nm of respective nitrogen sources by local isolate FNitrogen sourcesSoluble protein concentration (mg/ mL)24 hours48 hours72 hours96

hours120 hoursControl(chicken feather)0. 155 (±0. 009)0. 184 (±0. 007)0. 194 (±0. 008)0. 156 (±0. 004)0. 139 (±0. 008)Yeast extract0. 167 (±0. 006)0. 187 (±0. 009)0. 199 (±0. 007)0. 182 (±0. 005)0. 172 (±0. 007)Peptone0. 186 (±0. 009)0. 203 (±0. 009)0. 213 (±0. 007)0. 183 (±0. 005)0. 175 (±0. 011)Tryptone0. 193 (±0. 004)0. 193 (±0. 008)0. 218 (±0. 013)0. 212 (±0. 009)0. 195 (±0. 008)Soyabean cake0. 178 (±0. 006)0. 189 (±0. 012)0. 214 (±0. 010)0. 179 (±0. 012)0. 186 (±0. 015)Sodium nitrate0. 197 (±0. 006)0. 205 (±0. 005)0. 214 (±0. 008)0. 178 (±0. 006)0. 168 (±0. 007)*Values are expressed as mean ± standard deviation of three independent experimentsSoluble protein determination via Lowry method is an indirect measurement that can be used to investigate effect of nitrogen sources on keratinolytic activity for isolate F. This is because both inorganic and organic forms of nitrogen could be metabolized by most microorganisms to produce nucleic acids, proteins and cell wall components. (Kansoh et al., 2009). As shown in Table 4. 2, it appeared that the soluble protein for all nitrogen sources achieved the highest peak at 72 hours which was in conformation with the enzyme activity profile for isolate F. The complex mechanism of microbial keratinolysis basically involved synergic action between sulfitolysis and proteolytic system. Hence, the increased in soluble protein indicated that isolate F able to secrete keratinase enzyme to cleave peptide bonds in the waste chicken feathers which released substantial amounts of free sulfhydryl group, soluble protein and free amino acids in the fermentation broth. However, the trend of soluble protein profile for all the tested nitrogen sources does not really reflect much significant differences among each others in this study. Although medium supplemented with

tryptone was noticed to have the highest soluble protein, but it only increased slightly by 12 % when compared with the control. The same situation could also be observed in the media supplemented with the rest of nitrogen source. In this case, the range of the soluble protein was maintained within 0. 130 mg/ mL to 0. 220 mg/ mL. The result presented in Table 4. 2 was also found to be lower than the expected value in earlier research. For example, Jeong et al. (2010) on the study of Xanthomonas sp. P5 showed approximately 30% up to 70% increment in soluble protein concentration for medium added with tryptone, yeast extract, polypepeptide and sodium nitrate. The previous study reported higher value of soluble protein might be due to the researchers used the purified supernatant which had better stability and higher specific enzyme activity instead of whole cells fermentation for analysis. Besides, the free amino acid products generated from the hydrolysis of feather might be consumed back by the isolate F cells for growth and metabolism which could result in lower value of soluble protein than that of the reported value. Grazziotin, A., Pimentel, F. A., Sangali, S., de Jong, E. V., and Brandelli, A. 2007. Production of feather protein hydrolysate by keratinolytic bacterium Vibrio sp. kr2. Bioresource Technology. 98: 3172–3175