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Production of enzymes from pineapple crown and coffee husk by solid state fermentation

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Abstract. Agricultural waste had long become an environmental issue since there is no appropriate method for handling these residues. However, most of these wastes are used as animal feed or burned as an alternative for elimination in which it can cause air pollution. The main objective of this study is to produce enzymes from agriculture waste from pineapple plantation (pineapple crown) with coffee husk as co-substrate using solid state fermentation (SSF). The physicochemical properties (carbon, hydrogen, nitrogen, sulphur) of pineapple crown were characterized. During the SSF, the influence of three parameters that were co-substrate ratio, extraction ratio and incubation period were studied for the production of cellulase and bromelain enzymes. Titrimetric Method Assay and Filter Paper Unit Assay were used for the determination of bromelain and cellulase activity, respectively. Pineapple crown contained 39.50% carbon, 5.51% hydrogen, 13.82% nitrogen and 0.46% sulphur. These results proved that the effectiveness of pineapple crown as a substrate in solid state fermentation for production of the enzymes as it contains high C:N ratio (2.86). The optimum result for bromelain and cellulase activity were achieved when using co-substrate ratio 2:1 (w/w) with the presence of sludge, extraction ratio 1:5 (w/v) and incubation period of 4 days which are 1589 ± 9.89 GDU/g and 5.5851 ± 0.64 IU/mL respectively. Therefore, the production of hydrolytic enzymes (cellulase and bromelain) from pineapple crown can be accomplished by SSF, thus decreasing the operating cost.

Keywords: Solid state fermentation; pineapple crown; bromelain; cellulase

Introduction

Solid waste can be defined as useless and undesirable products which are in the form of solid resulting from human activities or being disposed by society. Solid waste can be divided into four categories which are organic waste, toxic waste, recyclable waste and soiled waste (Manaf et al., 2009). Examples of organic waste are agricultural waste, household food waste, human and animal waste. In this paper, we focused on agricultural waste which can be defined as unwanted by-products that have been generated from various kinds of agricultural operations.

Solid wastes have been a major environmental problem in Malaysia. Currently, over 23,000 tonnes of waste is produced each day in Malaysia (Guerrero et al., 2013). However, this amount is expected to rise to 30,000 tonnes by the year 2020. The amount of waste generated continues to increase due to the increasing population and development, and only less than 5% of the waste is being recycled (Guerrero et al., 2013). In addition, agricultural waste produced per year in Malaysia is about 998 million tons and it is estimated that 15% of the total waste generated in Asia are agro-waste (Ngoc and



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Schnitzer, 2009). Besides, pineapple harvested area in Malaysia was around 15,611 hectares, which produces 21.42 tonnes of pineapple fruits per hectare (Jaji et al., 2018). A lot of residue from pineapple planting is produced during harvesting activities in agricultural processes (Zainuddin et al., 2014). Pineapple waste comprised of core, peels, crown and extended stem. Thus, it is necessary to take the most convenient step to exploit these wastes into useable products.

Pineapple crown contains $2.41 \pm 0.08\%$ pulp, pH 3.94, total soluble solid of 1.6%, 0.30% acid, $0.83 \pm 0.04\%$ fructose and $0.51 \pm 0.01\%$ glucose. The presence of sugar in pineapple crown are suitable for the growth of microorganisms. Furthermore, pineapple crown also contained 53.4% holocellulase and 19.1% alpha-cellulase in which it will be further synthesized to produce cellulose (Tran, 2006). Hence, pineapple crown are the perfect substrate for production of enzyme by solid state fermentation since solid state fermentation (SSF) involves the growth of microorganisms on moist solid substrate in the absence of free water (Mitchell et al., 2006).

SSF has many advantages compared to submerged fermentation (SmF) such as the volumetric productivity eight times higher (Yazid et al., 2017). Besides, SSF also has easier downstream process than submerged fermentation and cost-effective. Solid state fermentation can produce a lot type of valuable products such as enzymes, aroma and flavour compounds, organic acids and protein-enriched agricultural residues for use as animal feeds (Yazid et al., 2017).

There are several measurable objectives have been derived to cater the aim of this study, that are to characterize the pineapple crown as a substrate for SSF, to determine the enzymes produced from SSF of pineapple crown and to determine the extraction ratio for enzyme production from SSF of pineapple crown. Therefore, in the present work, SSF is being introduced as one of the alternative method using pineapple crown which is obtained from agricultural waste as a substrate in the SSF process to produce different types of enzyme (cellulase and bromelain).

Materials and methods

Materials

Chemicals that were used in this study are acetic acid, glacial (CH_3COOH), 2.6M sodium chloride (NaCl), 1.0M sodium hydroxide (NaOH), gelatin from bovine skin, 3%v/v hydrogen peroxide solution (H_2O_2), 37wt % formaldehyde solution (CH_2O), 3,5 – dinitrosalicylic acid ($\text{C}_7\text{H}_4\text{N}_2\text{O}_7$), Rochelle salts ($\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$), 0.1N hydrochloric acid solution (HCl), citric acid monohydrate ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$) and glucose ($\text{C}_6\text{H}_{12}\text{O}_6$). All of these chemicals were purchased from Sigma-Aldrich. Pineapple crown was collected from Pekan Pina Sdn Bhd, Pekan, Pahang, while coffee husk was collected from Suraini Restaurant located in Gambang. Sludge was collected from Felda Panching Palm Oil Mill.

Preparation of substrate mixture for SSF

Pineapple crown was cut into small pieces with length of 2 inches each. Pineapple crown was mixed with coffee husk only as a co-substrate according to weight ratio of 0.5:1, 1:1 and 2:1 as a control for the SSF. For fermentation additional of 10% of volumetric ratio of sludge is added to the mixture of pineapple crown and coffee husk with weight ratio 0.5:1, 1:1 and 2:1 (w/w).

Solid state fermentation (SSF)

Solid state fermentation was carried out in 250 mL Erlenmeyer flasks in triplicate. It undergoes incubation for 96 hours at 37°C under static conditions at aerobic condition.

Enzyme extraction

Various amount of solid substrate to volume (mL) of distilled water was applied which are 1:3 and 1:5 (w/v). The solutions were stirred at magnetic stirrer for 30 to 45 minutes at room temperatures. Then, the liquid was be separated with the content in the flask and proceed to centrifugation at 10000 rpm, 4°C . The supernatant that is a crude enzyme was separated from the residue after centrifugation and stored at -80°C until further use. The crude enzyme was further analysed using different assay for cellulase and bromelain determination.

*Enzyme assays**Cellulase assay*

DNS Reagent and citrate buffer were prepared according to Lone et al., (2012). The total cellulase activity was determined using 50 mg Whatmann No. 1 filter paper strip by the standard IUPAC method (Ghose, 1987). Glucose (10 mg/ml) was used as a stock to prepare a standard curve. In a test tube add 1 ml of citrate buffer (pH 4.8) and 0.5 ml enzyme. A roll of filter paper strip (1 x 6 cm) was placed inside the solution and incubated for 1 hour at 50 °C. Then, 3 ml of DNS was added into the mixture and boiled for 5 min to deactivate the enzyme. Finally, 16 ml of distilled water was added prior to spectrophotometer reading at 540 nm. Test tube without addition of enzyme was treated as the blank. One unit (U) of cellulase activity was defined as the amount of enzyme required to liberate 1 μ mol reducing sugar from the appropriate substrate per min under the assay conditions.

Bromelain assay

The bromelain was determined by titration method (Krishnan and Gokulakrishnan, 2015). About 5% gelatin solution, 0.1N NaOH solution, 3% hydrogen peroxide solution, 37% formaldehyde solution were prepared accordingly. About 1 ml of crude enzyme was placed in a beaker and the pH is adjusted to pH 6 with 0.1N NaOH. Then, 25 ml of 3% gelatin was added and incubated in a waterbath at 45 °C for 20 min. Subsequently, 100 μ l of 3% hydrogen peroxide was added into the mixture and boiled for 5 min. The pH was adjusted to 6 by adding 0.1N NaOH. About 10 ml of 37% formaldehyde was added prior to adjusting the pH to 9 using 0.1N NaOH. Enzyme assay was done by titration of test and blank solution with 0.1N NaOH and gelatin as the substrate. One Gelatin Digestion Unit (GDU) is that amount of enzyme liberated after 20 minutes digestion at 45 °C.

Elemental characterization

Sample of pineapple crown was dried in an oven at 80 °C for 48h. Then the sample was grinded until become a fine powder. About 10 g of powdered pineapple crown was analyzed using CHNS elemental analyzer to find the component of carbon, hydrogen, nitrogen, and sulphur.

Results*Characterization of pineapple crown*

The elemental properties (C,H, N, S) of pineapple crown for enzyme production by solid state fermentation (SSF) are tabulated in Table 1.

Table 1: Summary of elemental properties (C,H, N, S) of pineapple crown

Element	Composition (%) dry basis
Carbon	39.504
Hydrogen	5.514
Nitrogen	13.818
Sulphur	0.459

Production of Bromelain from Pineapple Crown by SSF

This study was conducted to produce bromelain, a protease enzyme from crown of *Ananas comosus*. The pineapple crown was mixed with coffee husks and sludge according to the variation of weight ratio to give an appropriate substrate and microbiota during the fermentation. Others have proven that highest proteolytic activity and protein contents detected in the extract of pineapple crown (Ketnawa et al., 2012). The determination of bromelain activity is done by titrimetric assay method in which gelatin is used as the substrate. Gelatin was selected as substrate because it exhibits zone of clearance due to protease hydrolysis in higher amount when compared to other substrates (skimmed milk and casein)(Arumugam et al., 2017). From the enzyme kinetic studies, bromelain has strong affinity and

high catalytic efficiency towards gelatin when compared to casein (Kaur et al., 2015). Bromelain activity (GDU/g) from Day 1 to Day 4 of SSF for extraction ratio 1:3 and 1:5 (w/v) from different weight ratio of pineapple crown and coffee husk (2:1, 1:1, 0.5:1) are shown in Figure 1, respectively.

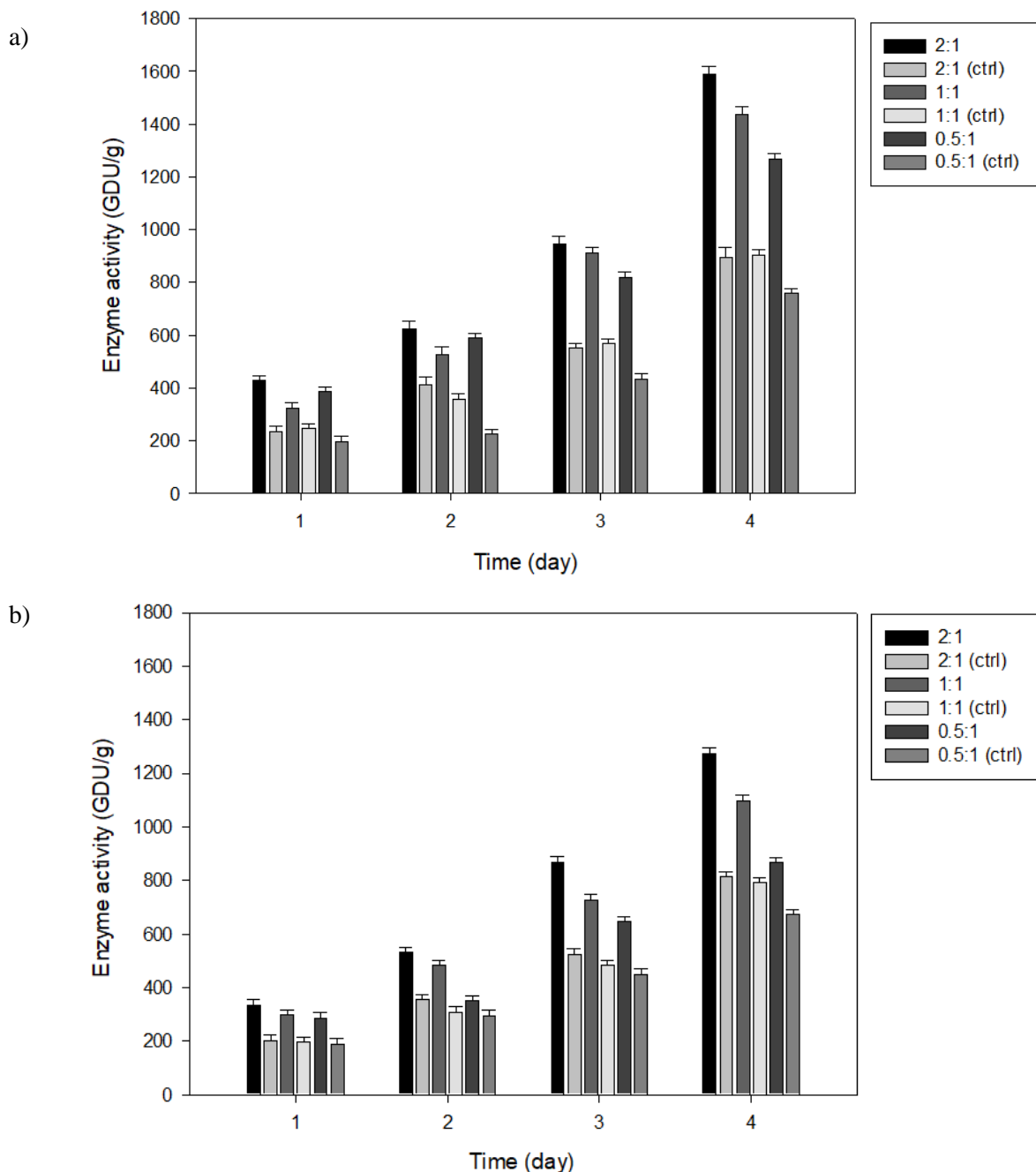


Figure 1: Bromelain Activity (GDU/g) during 4 days of SSF for extraction ratio of (a) 1:3 (w/v) and (b) 1:5 (w/v).

As can be seen in Figure 1, extraction ratio of 1:3 (w/v) exhibited more bromelain activity compared when using extraction ratio of 1:5 (w/v). Also, the addition of sludge in the mixture has enhanced the activity of the enzyme during 4 days of SSF.

Production of Cellulase from Pineapple Crown by SSF

Cellulase activity was measured by Filter Paper Unit (FPU) Assay – DNS Method. Cellulase activity (U/mL) from Day 1 to Day 4 of solid state fermentation process (SSF) for extraction ratio 1:3 and 1:5 for every co-substrate tested are determined. The data are illustrated in Figure 2.

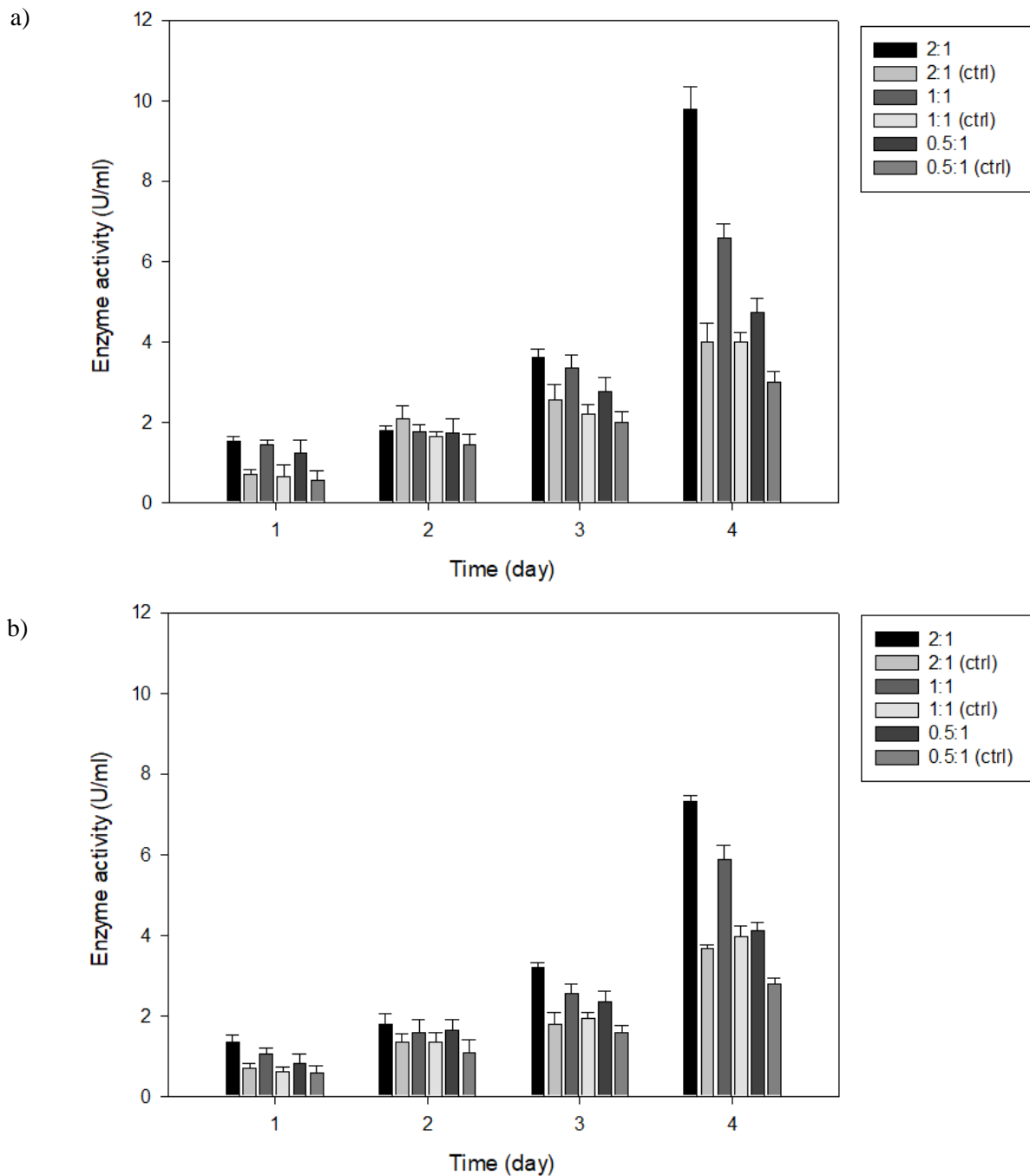


Figure 2: Cellulase Activity (U/ml) during 4 days of SSF with extraction ratio of a) 1:3 (w/v) and b) 1:5 (w/v)

As illustrated in Figure 2, day 4 of SSF produced the highest activity of cellulase for both extraction ratio. The production of cellulase increased with the addition of sludge with the highest weight ratio of pineapple crown and coffee husk (2:1 w/v).

The results of bromelain and cellulase activities are further discussed in discussion section based on co-substrate ratio, extraction ratio and fermentation duration.

Discussion

Effect of co-substrate ratio on enzymes activity

Pineapple crown and coffee husk were selected to study the effect of co-substrate ratio. These substrates are prepared in the ratio of 2:1, 1:1 and 0.5:1 (w/w). The total weight for co-substrate for every sample was standardized to 12g. Ten percent of sludge was added for co-substrate ratio 2:1, 1:1 and 0.5:1 to enhance the bromelain activity. From the three ratios tested, co-substrate ratio 2:1 produces the highest bromelain activity which is 1589 GDU/g and 1274 GDU/g for extraction ratio 1:3 and 1:5, respectively. Cellulase activity also at the maximum for substrate ratio 2:1 as illustrated in Figure 2. The result for cellulase activity is contradict from what has been reported by Selvam et al., (2014) in which highest cellulase production was obtained when coffee husk supplemented with pineapple waste in the ratio of 1 : 1. This differences may due to the addition of sludge as inoculum in co-substrate ratio 2:1 which cause the co-substrate to exhibit higher enzyme activities as stated in other study (Fadel, 2000). On the other hand, there is no report for the production of bromelain from the combination of pineapple crown and coffee husk as substrates for SSF.

Effect of extraction ratio on enzymes activity

After the samples undergo SSF, it was extracted with distilled water to substrate ratio of 1:3 and 1:5 (w/v). Based on Figure 1 and Figure 2, it has been proven that samples with extraction ratio 1:3 shows more enzyme (cellulase and bromelain) activity compared to 1:5 regardless of what type of co-substrate ratio is being tested. Since the crude extracts obtained is more concentrated in 1:3 compared to 1:5 (w/v). Theoretically, as concentration of crude enzyme increases, the rate of reaction (enzyme activity) also increases up to certain point. An enzyme is saturated when the active sites of all molecules are occupied, and at the saturation point, reaction will not speed up. The result is in accordance with the result obtained from Dubey et al., (2012) which revealed that maximum activity of the enzyme was found when the substrate concentration is at the highest. In contrast, Al-Sa'ady et al., (2016) reported that 1:2 (w/v) is the best ratio for bromelain extraction with specific activity 146.6 U/mg.

Effect of fermentation period on bromelain activity

To establish a successful fermentation process, it is necessary to choose best condition for high production of enzyme. As for this study, it is found out that four days (96 hours) of SSF is the best period to produce maximum amount of bromelain and cellulase. This period is selected as the highest production of enzyme according to the bromelain and cellulase activity profile showed in Figure 1 and Figure 2. The activity of bromelain increased with the increase in number of days of solid state fermentation process. For instance, as shown in Figure 1, which highlights the bromelain activity for extraction ratio 1:3, the activity of bromelain in Day 1 for co-substrate 2:1 is 427 GDU/g. This value increase steadily until it reached its optimum value at Day 4 which is 1589 GDU/g. Same goes with cellulase activity, the cellulase activity for 2:1 co-substrate ratio suddenly escalate to 5.5851 IU/mL in Day 4 from 2.2158 IU/mL in the previous day. Similar finding result also reported by Navya et al., (2012) in which 377.01 U/mL cellulase yield when it is incubated for 4 days. In contrast, Selvam et al., (2014) observed that the best incubation period for SSF is 2 and half day (60 hours) which resulted in 888 IU/mL cellulase. Theoretically, as the incubation period increase, the moisture content of the co-substrate will decrease hence increase the enzyme production. This is due to substrate porosity, alteration in substrate particle structure and increasing in oxygen transfer (Sahin and Arslan, 2008).

Conclusion

In this study, bromelain and cellulase have been extracted from co-substrate of pineapple crown and coffee husk by solid state fermentation (SSF). It can be concluded that pineapple crown is suitable for development of microorganism as it contains high amount of carbon. Co-substrate ratio 2:1 with the presence of sludge exhibit highest amount of cellulase and bromelain activity. 1:3 has been chosen as the best extraction ratio for combination of pineapple crown and coffee husk substrate to produce optimum activity for both enzymes (bromelain and cellulase) under study. It has been proven that 4

days is an excellent period for SSF which remarks that moisture content plays important role in fermentation process.

Further studies on the fermentation day can be prolonged to see whether after 4 days the production of enzyme is still increasing or decreasing. Also different types of parameter used such as pH, temperature, type of buffer used for extraction could be helpful to identify and better analyze the potential of cellulase and bromelain under its best operating condition. Besides, other agriculture waste such as sugarcane bagasse, wheat straw and others also can be exploited and used as a substrate in SSF as it seems to be a promising strategy to produce enzyme at lower cost and at the same time reduce the amount of waste generated.

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