CHAPTER 5

DISCUSSIONS

5.1 Components of sugar depleted dried longan (SDDL)

Ground SDDL in this study contained sucrose, glucose and fructose (Table 4.1). Normally, the main component in dried longan is carbohydrate about 72.70% which composed of glucose, fructose and sucrose (Angkasit *et al*., 1999). Moreover, the amount of sugars decreased when dried longans were stored for many years. Agustina (2009) reported that 10 months old and 6 years old dried longan of the same cultivar as our study (*Dimocapus longan* Lour. cv. Daw) consisted sucrose about 3.1870 g/g and 0.0005 g/g and glucose 0.5267 g/g and 0.0017 g/g and fructose about 1.5350 g/g and 0.0000 g/g, respectively. However, the amount of sucrose, glucose and fructose content in dried longan in this study were slightly higher than those of the previous reports.

The sugars in deadstock dried longan reported by Agustina (2009) were too low to be used as a substratre for ethanol production by yeast. Agustina (2009) reported that the 6 years old dried longan which contained small amount of sugar can produce a tiny amount of ethanol by *C. utilis* TISTR 5001which yielded only 1.26 ± 0.82 g/l. Hence, SDDL is not an appropriate substrate in direct ethanol production. Acid hydrolysis, microbial treatment and enzymatic hydrolysis should be employed in order to produce reducing sugar for ethanol production by yeast (Abedinifar *et al*., 2009; Dias *et al*., 2010; Karimi *et al*., 2007; Lu *et al*., 2009; Patel *et al*.,2007).

Dried longan pulp had moisture and carbohydrate which mainly composed of three sugars including glucose, fructose and sucrose (Angkasit, 1999; Panyatep, 2005) and it also contained fiber around 1.6%. Therefore, if dried longan was stored for long time, the microbes may contaminate and consume sugar in dried longan then fiber became main component of dried longan instead. Sumphanwanich (2006) reported

that main component of longan pulp pomace is cellulose, which was about 78.36 % (w/w). Furthermore, longan pulp also contained pectin about 0.20 g/100 g fruit (Mahattanatawee *et al*., 2006). In the case of longan pericarp, Jaitrong *et al*. (2007) reported that longan pericarp had total dietary fiber, lignin and pectin about 7.26, 0.89 and 0.019 g/100g dry weight respectively. Longan seed contained starch as high as 40-60% of weight (Lai *et al*., 2000; Viet *et al*., 2005). Visuthithada (2008) reported that longan seed had fiber about 54.30 % dry weight basis. These components (cellulose, hemicelluloses, lignin, pectin and starch) were found in SDDL used in this study (Table 4.2). However, the amounts of these components in this study were not similarly to other researches because dried longan used in this study was stored for a long time in godown without humidity and temperature control. These dried longans were contaminated with microorganisms such as fungi which can hydrolyze these component for their growth. In general, the lowest water activity (aw) and temperature for fungal growth are 0.65 and 4ºC, respectively (Baxter and Illston, 1980; Hudson, 1986).

5.2 Enzyme activities from fungal fermentation

Five fungus strains (*Aspergillus niger* TISTR 3063 and 3089, *A. foetidus* TISTR 3461 and *Trichoderma reesei* TISTR 3080 and 3081) were selected to produce reducing sugar from SDDL. After SDDL was fermented by these fungi, enzyme activities (cellulase, xylanase, mannanase, pectinase and amylase) were observed (Figure 4.1, 4.2, 4.3, 4.4 and 4.5 and Table 4.3). The maximum enzyme activities were likely to depend on the strain of fungi. *A. niger* TISTR 3089 showed maximum mannanase and xylanase activities and *A. niger* TISTR 3063 showed maximum cellulase activity. Several research groups mentioned about the use of *A. niger* to produce cellulase, xylanase and mananase. For example, Acharya *et al*. (2008) used saw dust pretreated by 2 N NaOH as a substrate to produce cellulase by *A. niger* at various condition, the maximum cellulase activity obtained was 0.1528 IU/ml. Sae-lee (2007) mentioned that *A. niger* can produce mannanase, cellulase and xylanase by using palm kernel meal as a substrate, the maximum enzyme activities were 20.2, 1.3 and 3.2 U/g koji respectively. Yuan *et al*. (2005) used *A. niger* to investigate the effect

of temperature shift on the production of xylanase, the highest xylanase activitiy was 290 U/ml.

For *A. foetidus* TISTR 3461, high pectinase and amylase activities were observed. There were many studies reported that *A. foetidus* can produce pectinase and amylase. Chatanta *et al.* (2008) used *A. foetidus* MTCC 151 as a pectinase producer, and used *Fusarium oxysporum* MTCC 1755 (cellulase producer) and *Saccharomyces cerevisiae* as mix culture to ferment waste apple pomace for ethanol fermentation. Jagadeeshbabu and Viswanathan (2010) *used A. foetidus* (NCIM 505) to produce pectinase by using tamarind kernel powder as the substrate in submerged fermentation at various pH, temperature and metal ions. Michelena and Castillo (1984) reported that *A. foetidus* ATCC 10254 can produce amylase using rice flour as a substrate. Tongklib (2005) reported the production of α-amylase by *A. foetidus* ASKU21 using rice bran as a substrate and fermented for 5 days, amylase can be produced up to 115 U/g dry substrate.

Moreover, the amount of enzyme may depend on composition of substrate. Okafor *et al*. (2007) used *A. niger* ANL301 to produce xylanase on various agro-wastes i.e. sawdust, sugarcane plup and wheat bran, the maximum xylanase activities were 0.65, 0.95 and 6.47 U/ml, respectively. Kunpratum (2000) reported that wheat bran and sugarcane pulp had xylan about 26.10 and 20.31 g/g dried substrate. Therefore, the value of enzyme activity is likely to depend on the components of substrate. However, the enzyme cellulase and hemicellulase activities reported here were not correlated with the high cellulose and hemicellulose content in SDDL (Table 4.2). This result indicated that cellulase and hemicellulase production was inhibited. There were studies reported that the ability to produce cellulase and xylanase of fungi were inhibited when glucose concentration exceeded 0.1% (Simao *et al*., 1997; Saddler and Mes-Hartree, 1984). Sepahy *et al*. (2011) mentioned that adding 1% of glucose into substrate can decrease xylanase activity from 252.722 IU/ml (control) to 14.7282 IU/ml. Therefore, it is believed that fungi hydrolyzed starch into glucose and inhibit cellulase and xylanase expression.

In order to increase enzyme activity, some supplements were added into SDDL before fermentaition. Kheng and Omar (2005) and Sepahy *et al*. (2011) reported that adding of suitable nitrogen source concentration into agricultural wastes

can increase enzyme activity. Furthermore, adding of some nitrogen sources such as peptone, yeast extract, $NaNO_3$, NH_4NO_3 and metal salts such as $MgSO_4$ or adding of amino acid such as asparginine can increase amylase activity to be higher than others supplements (Muhammad *et al*., 2012). Therefore, the supplementation of suitable nitrogen sources and/or some supplements in SDDL would improve enzyme production.

5.3 Screening of 5 strains of fungi for maximum reducing sugar production

Reducing sugar was measured by DNS method during 10 days cultivation. The maximum reducing sugar yield was obtained from *A. foetidus* TISTR 3461 at 30.84 ± 1.02 g/l or 102.79 ± 3.39 g/g (Table 4.4). There are many studies which produced reducing sugar from agricultural residues by filamentous fungi. For example, Patel *et al*. (2007) reported that the highest reducing sugar yields from wheat straw treated by *A. awamori*, rice straw treated by *A. niger* and *A. awamori*, rice husk treated by *T. viride* and bagasse treated by *P. chrysosporium* were 18, 16, 22.5 and 90.5 mg/g, respectively. Prasad (2008) studied about the pre-treatment of some agro-residues by many fungi and found that the maximum total sugars obtained from sugarcane bagasse treated by *T. viride*, paddy straw treated by *T. viride* and wheat straw treated by *A. sidowia* were 31.45, 26.41 and 28.55 mg/g, respectively. In 2009, Arbsuwan hydrolysed SDDL pomace with cellulase and pectinase and obtained sugar around 30 – 35 mg/g longan pomace.

Higher reducing sugar content reported in the present study might be the result of high content of starch (Table 4.2). Many agricultural wastes which popularly used for producing reducing sugar with microbial treatment process such as sugarcane bagasse, wheat straw, rice straw, rice husk, were observed to contain cellulose, hemicelluloses and lignin called lignocelluloses as the main components (Han *et al*., 2012; Lee, 2005; Luduena *et al*., 2011; Mohan *et al*., 2012). Therefore, the amount of reducing sugar content in this study was higher than other researches because starch can be easier digested than cellulose since all starch's glucose units orient in the same direction (Nasr, 2009; Panyamara and Panyamara, 2011; Wasserman, 2011). On the contrary, adjacent glucose unit in cellulose is rotated 180 degrees around the main

polymer axis. This makes cellulose water-insoluble and has a high tensile strength (Anderson, 2002; Glazer and Nikaido, 1995). Therefore, it is much more resistant to be degraded than starch.

Some researchers used cassava wastes which contains starch as main component to produce reducing sugar. Fungsin *et al*. (2007) reported the fermentation of cassava wastes containing cellulose, hemicellulose and starch at levels of 24.99, 6.67 and 61% (w/w) respectively with *Aspergillus niger* TISTR 3352 without any supplementation. The maximum reducing sugar at 99.8 mg/g was reported which was lower than this study.

However, the higher yields than this study were also reported. Chumkhuntod (2000) reported that the degrading of cassava by many yeast and fungal strains was produced reducing sugar range between 17 - 560 mg/g and many strains cultures had higher reducing sugar yield than the current study. Yuwa-Amornpitak (2010) reported that *Rhizopus* sp. #s3Su produced reducing sugar from cassava at 259 mg/g approximately which higher than this study as well. Therefore, the ability to produce reducing sugar may depend on the strains of the microbes used. The reason that this study got lower reducing sugar than above reports may be caused by lignin presented in dried longan. Berlin *et al*. (2006) reported that enzyme activities of cellulases and xylanases can be inhibited by lignin. SDDL in this research had lignin content about 24.24% (w/w). Cassava contains starch as main component about 67% but it has a little fiber (which consist of lignin) about 3.9% - 4.4% (Sauvant *et al*., 2004; Suksombat *et al*., 2007; Tonukari, 2004). Therefore, it has less lignin content than SDDL. The other inhibitor in the SDDL is tannin which was found in longan seed around 4.4 – 9.9% (Viet *et al*., 2005; Visuthithada, 2009, Zheng *et al*., 2009). It can inhibit amylase activity and fungal growth (Huynh *et al*., 1992; Komninos *et al*., 1988; McDougall *et al*., 2005; Nichols-orians, 1991). Hwang *et al*. (2001) reported that tannin can inhibit the synthesis of chitin which is a component of fungal cell wall. Consequently, fungi which were cultured on substrates with high content of lignin and tannin may yield less reducing sugar.

The addition of nitrogen supplement can increase ability of enzyme production (Oshoma *et al*., 2010; Varalakshmi *et al*., 2009). Fungsin *et al*. (2007) reported that cassava wastes which the supplement of 0.02% (w/v) ammonium sulfate was added

and treated by *A. niger* had reducing sugar yield about 124.89 mg/g which higher than without added supplement about 99.8 mg/g. In case of SDDL in this study, there was no supplement added before fermentation.

Apart from nitrogen source addition, reducing sugar content may be higher if acid and/or enzyme hydrolysis are employed after fermentation (Baba *et al*., 2011; Margeot *et al.*, 2009). However, using acid and enzyme hydrolysis can increase the cost of ethanol production (Demirbas, 2005; Woiciechowski *et al*., 2002).

5.4 Ethanol production from fungal treatment extract

S. cerevisiae TISTR 5606 can grow and produce ethanol from SDDL treated by *A. foetidus* TISTR 3461 and *A. niger* TISTR 3089 extracts. The diminution and the amount of fermentable reducing sugar from *A. foetidus* TISTR 3461 extract and *A. niger* TISTR 3089 extract fermentation were correlated with glucose consumption and glucose concentration (Figure 4.8 and Figure 4.9). Hence, the fermentable reducing sugar can be assumed as glucose. Therefore, glucose was the main reducing sugar received from microbial treatment process of SDDL. Glucose in both fungal extract may be obtained from the hydrolysis of starch by both fungi which related to higher amylase activity. The fermentable reducing sugar obtained from both extract was 65.71 ± 2.09 % and 57.41 ± 6.41% (w/w) (Table 5.1). Therefore, the glucose production of both fungal strains from starch in SDDL was 318.68 ± 9.16 mg/g starch and 225.01 ± 29.11 mg/g starch, respectively.

Furthermore, it should be noted that not all reducing sugars from fungal extract are fermentable sugars. There was reducing sugar left in the culture broth at the end of ethanol fermentation. Therefore the extract must contain other sugars (non fermentable reducing sugar). Table 5.1 compares the non-fermentable sugar and fermentable sugar in substrate before fermented by yeast. Figure 5.1 reveal that there was an unknown peak within the glucose peak (at retention time 7.034 min) which was clearly seen at the end of fermentation. This unknown peak may be galacturonic acid because the retention time of galcturonic acid and glucose were close when using HPX-87H column with 0.005 M H_2SO_4 as mobile phase (Figure 5.2). Galacturonic acid is the main structure in pectin (Schols and Voragen, 1996). Therefore, pectin

can be hydrolyzed by pectinase to release shorter molecules of galacturonic acid (Shatty *et al*., 2006). *A. foetidus* TISTR 3461 and *A. niger* TISTR 3089 used in this study can produce maximum pectinase activity in SDDL at 14.59 U/gds and 8.05 U/gds, respectively (Figure 4.4 and Table F.4). Therefore, galacturonic acid can be found in both fungal extracts. Moreover, *S. cerevisiae* cannot consume a galacturonic acid and its growth also can be inhibited by galacturonic acid (Huisjes *et al*., 2012).

Table 5.1: Comparison of fermentable reducing sugar and non-fermentable reducing sugar content in fungal extracts.

	Fermentable reducing sugar (% (w/w))	**Non-fermentable reducing sugar (% (w/w))**
A. foetidus TISTR 3461 extract	65.71	34.29
A. niger TISTR 3089 extract	57.41	42.59

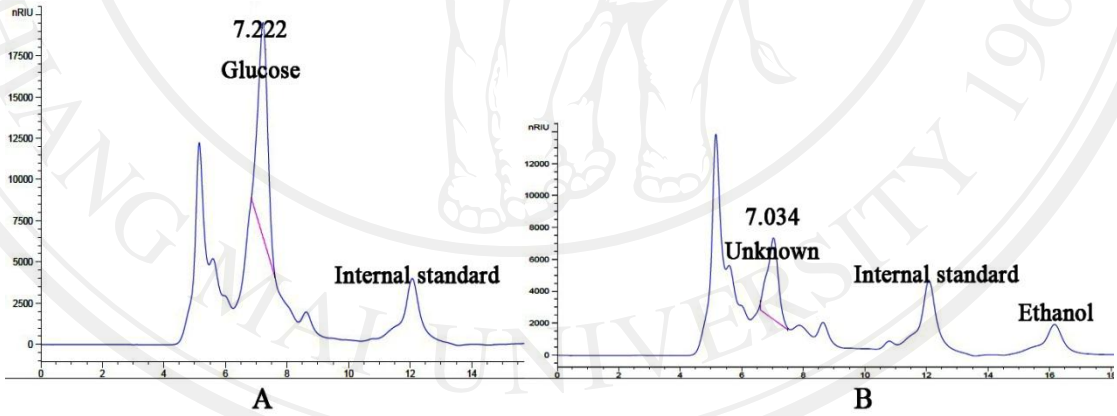

Figure 5.1: Chromatogram from *A. niger* TISTR 3089 extract before (A) and after (B) *S. cerevisiae* TISTR 5606 fermentation.

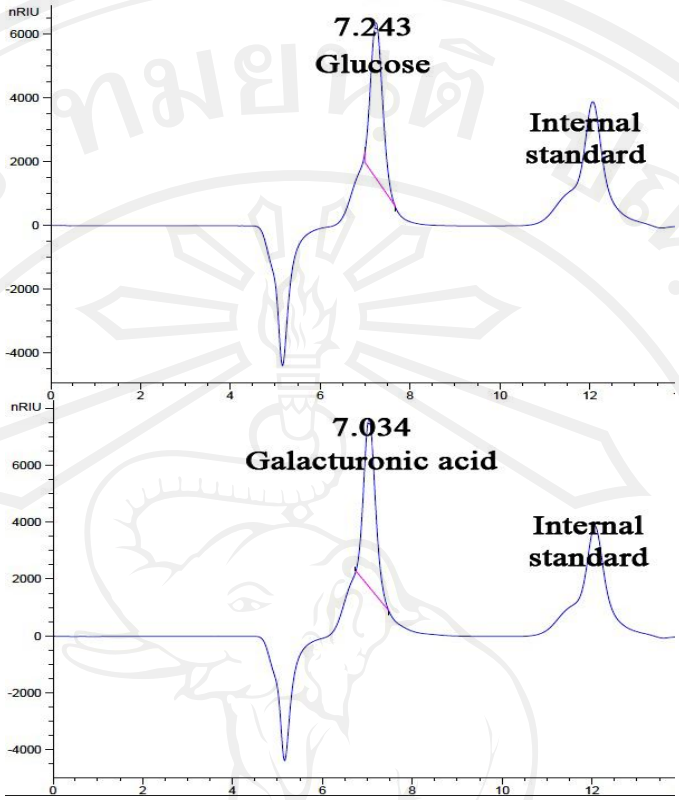

Figure 5.2: Chromatogram of glucose and galacturonic acid.

Agustina (2009) used 6 years dried longan that contained total sugar at approximately 2 g/l to produce ethanol by *Candida utilis* TISTR 5001 and reported that the maximum ethanol content was only 1.26 g/l. To compare with ethanol production from Agustina (2009), there was a study showed that the ethanol production yield was not significant change with different of initial sugar concentration (Marx *et al*., 2012). Therefore, the maximum ethanol production yield from using concentrated *A. foetidus* TISTR 3461 extract was 0.41 ± 0.003 g/g should be equal to the non-concentrated fungal extract. If the extract from fungal without concentration (reducing sugar around 30.84 ± 1.02 g/l from *A. foetidus* TISTR 3461) was used to produce ethanol, ethanol production would be 12.3 g/l. Therefore, the pretreatment of low sugar substrate with microorganisms is an option to increasing ethanol production.

The ethanol production from *A. niger* TISTR 3089 extract and *A. foetidus* TISTR 3461 extract were higher than other studies because they did not concentrate the extract from fungal treatment of agricultural wastes before ethanol fermentation.

Gurav and Geeta (2007) studied ethanol production from fungal pretreatment of agro residues by yeasts and *Zymomonas mobilis*. They reported that the maximum ethanol concentration was obtained from *Z. mobilis* in paddy straw, wheat straw and bagasse pre-treated, they were 588.7 mg/l, 638.4 mg/l and 820.8 mg/l, respectively. Patel *et al*. (2007) studied the ethanol production from microbial pretreated agricultural residues. They reported that the maximum ethanol production from *S. cerevisise* (NCIM3095) was found in bagasse, pre-treated by mixing of *A. awamori* and *Pleurotus sajor-caju*, it was about 9.8 g/l. Therefore condensation of reducing sugar from fungal treatment can increase initial sugar concentration and maximize ethanol production by yeast.

The ethanol productions from both fungal extracts were lower than that obtained from YMB and SDDL with 100 g/l glucose (Table 4.7). This can be explained by the lower initial glucose concentration than others substrate (Figure 4.8). However, the ethanol production yields from the both fungal extracts were similar to YMB and SDDL without fungal treatment (Table 4.8). Therefore, the difference of initial glucose concentration did not affect the ethanol production yield even though it influenced maximum ethanol production as reported previously by Marx *et al*. (2012).

The specific glucose consumption rates and specific ethanol production rate from both fungal extracts were similar to YMB but lower than SDDL extract without fungal treatment with glucose (Table 4.6 and 4.7). At 12 h-cultivation, *S. cerevisiae* TISTR 5606 in SDDL extract without fungal treatment with glucose had lower dried cell weight than cultured *S. cerevisiae* TISTR 5606 in YMB (Table I.1). This can be explained that SSDL extract may have some inhibitor which retard yeast growth. Tannin may be one inhibitor which retard *S. cerevisiae* TISTR 5606 growth rate because it was found in longan seed (Viet *et al*., 2005; Wauters *et al*., 2001).

The difference in dried cell weight (Table 4.5), glucose consumption rate (Table 4.6), and ethanol production rate (Table 4.7) using both fungal extracts as substrate with others control substrates may be the result of the content of supplement in each substrate. The addition of nitrogen sources in the substrate can increase biomass production, the consumption rate and the production rate of fermentation (Beltran *et al*., 2005; Turhan *et al*., 2010). Moreover, Arrizon and Gschaedler (2002) reported that adding nitrogen sources was important to fermentation efficiency such as reduction in fermentation time and increasing the maximum sugar consumption

rate. In this study, glucose solution culture had the lowest glucose consumption rate, ethanol production rate and also yeast dried cell weight. This indicated that fungal extract contained some supplement which improved yeast fermentation efficiency. This supplement came from SDDL as indicated by the result from using SDDL without microbial treatment extract with 100 g/l glucose as substrate, which had higher glucose consumption rate, ethanol production rate, and dried cell weight than using both fungal extracts as substrate. The lower rates from fungal extract may be explained as some supplement was used by fungi during microbial treatment process because nitrogen is important for the growth of fungi by supporting nucleic acid, protein and enzyme production (Barron, 2003). Moreover, YMB culture, rich in nitrogen source, was the highest in ethanol production rate and dried cell weight.

Although each substrate contained different level of nitrogen source, the final ethanol yield did not differ markly (Table 4.8). This correlates well with results from other research teams. For instance, Hong and Yoon (2011) reported that adding nitrogen supplement can increase the ethanol production rate however the ethanol production yields from food residue with and without nitrogen source were equal at final fermentation. Moreover, Ruanglek *et al*. (2006) studied the ethanol production of *Zymomonas mobilis* NRRL-B-14023 from substrate which composed of variant nitrogen concentration *via* control medium (contained high supplement content), ami-ami solution, brewer's yeast autolysate and fish soluble waste and reported that there were no significant different between ethanol production yield (around 0.45 g/g) at 24 h fermentation.

In this study, the ethanol production yield ($Y_{p/s}$) was lower than the theory (0.51 g/g). It may be possible that fermentation condition was the cause as explained by several groups. The non-agitation fermentation used in this study may lower ethanol production yield. Rodmui *et al*. (2008) studied the agitation conditions for maximum ethanol production by coculture of *S. cerevisiae* and *Candida tropicalis*, they reported that the maximum ethanol concentration, the ethanol productivity and the ethanol yield in the cultured with the agitation speed was 50 rpm were higher than with static condition and with faster agitation. The agitation could be advantageous to the growth and performance of microbial cells by mixing the medium components in the culture to improve the mass transfer characteristics, such as oxygen transfer rates

with respect to substrate and product/by-product (Rodmui *et al*., 2008). Moreover, in static condition, the yeast cells would be settled to the bottom of the culture, therefore they could not absorb nutrient well. As the result, the biomass was so low and the cell size was so small that they were weak in ethanol production (Liu *et al*., 2009). Furthermore, adding aeration during the initial stage of yeast growth along with agitation increased the final ethanol concentration from 128.1 g/l (without aeration) to 143.8 g/1 (Liu *et al*., 2009). The insufficiency of oxygen could limit growth, decreased yeast viability resulting in slow and incomplete fermentation (Cysewski and Wilke, 1976). Therefore, the supplement of adequate aeration during initial ethanol fermentation can improve the ethanol production yield.

Moreover, another possible cause, furfural and hydroxymethyfurfural generated during the heat-pretreated of pentoses and hexoses can lead to low ethanol prodcution (Modig *et al*., 2002; Taherzadeh, 1999). Wikandari *et al*. (2010) reported that addition of 0.5 g/l and 1.0 g/l of furfural and hydroxymethylfurfural can decreased ethanol yield and productivity. However, in this study, the furfural might not be affect fermentation efficiency sine the ethanol production yield from sterilized extract and non-sterilized extract were not different (Table 4.9).

5.5 Comparison of sterilized and non-sterilized extract on ethanol production

After sterilization, the sterilized extract had more aggregate than non-sterilized extract (data not shown). However, after ethanol fermentation, there was no significant different on ethanol production between sterile and non-sterile substrate. However, although the ethanol production yields at maximum production time were different ($p \leq 0.05$), they had a little bit different (Table 4.10 and Table 4.12). Therefore, the results from this experiment implied that the non-sterile extract from both strains of fungi is suitable for ethanol production. The obtained result also indicated that in the non-sterile extract, *S. cerevisiae* can produce ethanol even though fungus was contaminated in substrate. Ado *et al.* (2009) reported that ethanol production around 6.17% was obtained from co-culture fermentation of *S. cerevisiae* and *A. niger* with 10% corn cob. Jeon *et al*. (2008) reported that ethanol produced from 50 g potato starch/l by a mixed culture of *A. niger* and *S. cerevisiae* was about

5 g/l in a conventional bioreactor. These suggested that the extract may not be prior sterilized for the ethanol production. The advantages of the non-sterilization of the extract are lower equipment requirement and energy consumption which can reduce the cost (Qin *et al*., 2009). The cost in this study was reduced around 6 baht for each sterilization by autoclaving (Autoclave model ACV-3167 with power 3000 w). Moreover, using non-sterilized substrate may reduce the problem from the Maillard reaction, which leads to the production of unfavorable furfural in sterilization process (Banerjee *et al*., 1981).

5.6 Comparison of pH adjustment of the extract on ethanol production

The initial pH value in SDDL was 5.05 ± 0.01. After fermentation by *A. foetidus* TISTR 3461 and *A. niger* TISTR 3089, the pH value was decreased to 3.92 ± 0.03 and 2.54 ± 0.04, respectively. This can explain by acid production especially citric acid production of both fungi (Chen, 1993; Majumder *et al*., 2010). Moreover, citric acid production is increased if the substrate contains high content of starch. (Vandenberghe *et al*., 2000).

However, in the case of the extract from SDDL treated by *A. foetidus* TISTR, after fermented by yeast, there was no different in ethanol production yield from substrate with pH adjustment (Table 4.13 and 4.14). This supported by the optimum range of pH value for yeast growth and ethanol production was 4 to 6 (Blank and Sauer, 2004; Narendranath *et al*., 2005). Moreover, Benson (2004) reported that the limits of pH for growth of *S. cerevisiae* were 2.4 and 8.6.

In the case of *A. niger* TISTR 3089 extract the pH was low as 2.54 (Table 4.15), *S. cerevisiae* TISTR 5606 cannot growth on this pH because the pH was out of optimum range. Thomas *et al*. (2002) reported that *S. cerevisiae* had poor growth when cultured on media which had pH below 2.5. Therefore, the pH of A. *niger* TISTR 3089 extract must be adjusted prior to fermentation.