Maximizing lipid accumulation from molasses by *Trichosporon asahii* and effect of oleaginous yeast supplementation in dairy ration on *in vitro* nutrient digestion

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ABSTRACT: In this study, a complete randomize design was used to investigate the effect of molasses concentration, $(NH_4)_2SO_4$ concentration and culture conditions on lipid accumulation in *Trichosporon asahii* GSY10. The results showed that favorable molasses and $(NH_4)_2SO_4$ concentration in culture media were 120 and 0.5 g/l. Optimum initial pH, cultural temperature and cultural time were 5, 30 °C, and 8 days, respectively. Under this condition, lipid yield and lipid content were 4.1 g/l and 39.5% of dry biomass, which increased to become higher than the original values (2.6 g/l and 23.5% of dry biomass). In the subsequent study, five total mixed ration (TMR) containing *T. asahii* GSY10 supplementation at levels 0, 4.0, 8.0, and 12.0% of DM compare to palm oil at level 3.0% of DM were arranged in completely randomize design in order to examine effect of oleaginous yeast supplementation on nutrient digestions. The results of *in vitro* study illustrated that oleaginous yeast supplementation did not affect *in vitro* dry matter digestibility (IVDMD) ($P > 0.05$). However 8% of its supplementation tended to improve fiber digestion ($P < 0.1$). Palm oil supplementation markedly decreased IVDMD when compared with others ($P < 0.05$). From the information gained the *in vitro* study, oleaginous yeast has a potential use as alternative fat source in ruminant diet.
Keywords: cellular lipid, molasses, oleaginous yeast, ruminal digestion, *Trichosporon asahii*.

Introduction

Oleaginous yeasts refer to yeasts that are able to accumulate lipid in their cell more than 20% of dry biomass (Beopoulos et al., 2009). Currently, there is a great attention on oleaginous yeast because they can grow on agricultural and industrial residues and their lipid composition is similar to plant oil (Huang et al., 2009; Liang et al., 2010). Several researches reported that lipid from oleaginous yeast could be used for bio - diesel production (Angerbauer et al., 2008; Zhu et al., 2008) and as cocoa butter replacer for chocolate production (Papanikolaou et al., 2003). In ruminant nutrition, feeding yeast cell provided protein and vitamins for rumen microbes and host animal (Steckley et al., 1979) as well as maintained ruminal pH and stimulated growth of fiber - degrading bacteria (Fonty and Chaucheyras - Durand, 2006). However, there has been limited information of oleaginous yeast supplementation in ruminant ration. Jalč et al. (2009) illustrated that microbial lipid supplementation showed the improving of ruminal fermentation and degradation of cellulose and hemicelluloses. Oleaginous yeast production for animal feed may be an alternative approach for protein and lipid supply as well as improving of ruminal digestion.

Molasses, by - product from sugar industrial have been used in microbial lipid production in

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order to reduce the cost of production (Johnson et al., 1995). This substance contains approximately 45% fermentable sugar which is a good carbon source for microbial lipid production (Wu et al., 2011). In our previous study, oleaginous yeast, *Trichosporon asahii* GSY10 was isolated from soil and this strain accumulated lipid at level 23.5% of dry biomass when using molasses as carbon source and $(NH_4)_2SO_4$ as nitrogen source (Paserakung et al., 2015). Karatay and Dönmez (2010) demonstrated that lipid accumulation in oleaginous yeast could be improved by optimizing of carbon and nitrogen concentration. Additionally, culture condition such as initial pH, cultural temperature and time had markedly affect lipid accumulation in oleaginous yeast (Zhu et al., 2008). Therefore, the objectives of the study were to improve lipid accumulation from in *Trichosporon asahii* GSY10 by optimizing of molasses and $(NH_4)_2SO_4$ concentration as well as culture conditions, and to evaluate the effect of oleaginous yeast supplementation on *in vitro* nutrient digestion of TMR.

Materials and Methods

Microorganism and pre - cultivation

Trichosporon asahii GSY10 was obtained from our laboratory. This strain was isolated from grassland soil at Roi - Et Dairy Research and Training Center, Roi - Et province, Thailand, and was identified by comparing 26S rDNA sequence with the sequences of Genbank databases (Paserakung et al., 2015). Seed culture was prepared by transferring one loop full of *T. asahii* GSY10 into 50 ml of media containing (in g/l): glucose 40, yeast extract 15, peptone 5, with adjust pH onto 6 (Dai et al., 2007) and then incubated in incubator shaker at 30°C with shaking at 150 rpm for 24 h.

Optimization of carbon and nitrogen concentration in culture medium

The 3x3 factorial experiment arranged in a completely randomize design was conducted to determine the effect of molasses and $(NH_4)_2SO_4$ concentration on lipid accumulation in *T.asahii* GSY10. Culture medium contained 80 g/l molasses, 1.0 g/l $(NH_4)_2SO_4$ and 0.5 g/l KH_2PO_4 (Karatay and Dönmez, 2010) with modification by increasing molasses concentration from 80 to 120 and 160 g/l and $(NH_4)_2SO_4$ concentration from 0.5, 1.0, and 1.5 g/l, respectively. Seed culture (5 ml, average 5.2 x 10^7 cell/ml) was inoculated into 45 ml of culture medium (pH 5) and then incubated at 30°C with shaking at 150 rpm for 6 days.

Determination of optimum initial pH, cultural temperature, and cultural time

In order to determine the effect of initial pH value on lipid accumulation of *T. asahii* GSY10, three different initial pH values (4, 5, and 6) were assigned to the culture. Cultural temperature was kept on 30°C and cultural time was 6 days.

Subsequently, the effect of cultural temperatures on lipid accumulation of *T. asahii* GSY10 was determined by varying cultural temperatures from 25°C to 30°C and 35°C. Initial pH value was also adjusted onto 5 and cultural time was 6 days.

Finally, the effect of cultural time on lipid accumulation of *T. asahii* GSY10 was examined by increasing the cultural time from 2 to 4, 6, 8, and 10 days. The initial pH value was 5 and cultural temperature was 30°C.

All of the experiments mentioned above, cultures were incubated in orbital shaker withshaking at 150 rpm. The treatments were arranged in completely randomize design (CRD).

Biomass and lipid analysis

Biomass was harvested by centrifugation and dried at 105°C for 24 h (Johnson et al., 1995). Lipid was extracted using Bligh and Dyer method with modification described by Pan et al. (2009). The dried yeast was suspended in 10 ml of 4 M of HCl and incubated at 60°C for 1 h to 2 h. Acid - hydrolyzed samples were mixed with 20 ml of chloroform/methanol mixture (1:1 v/v) for 2 - 3 h. The lower phase containing lipids was recovered and evaporated in 40°C water bath under N_2. The dried lipid was then weighed.

In vitro ruminal digestion

Experimental diets containing total mixed ration (TMR) supplemented with 0, 4.0, 8.0, 12.0% of DM of *Trichosporon asahii* GSY10 slurry and TMR supplemented with 3.0% of DM of palm oil were arranged to completely randomize design (CRD) with five replicated. TMR consisted of 40% Napier silage and 60% concentrate mixed **(Table 1)**. *In vitro* ruminal digestion activity was evaluated using the method as described by Tilley and Terry (1963) with slightly modification. A mass of 0.5 g of ground samples (2 mm) were weighed into 130 ml septum bottles, then filled with 67 ml of McDougall' saliva buffer containing (in g/l): 9.8 $NaHCO_3$, 2.77 Na_2HPO_4, 0.57 KCl, 0.47 NaCl, 0.12 $MgSO_4.7H_2O$, and 0.16 $CaCl_2.H_2O$. Then, the mixture was add with 33 ml of strained ruminal fluid collecting from two dry cows using sterile esophagus tube with vacuum 2 h after morning feed. Samples were flooded with CO_2, capped and placed in incubator shaker and incubated at 39 °C for 48h. After that, samples were dried at 55ºC for 72h. Residues were analyzed for NDF and ADF according to the method described by Van Soest et al. (1991). *In vitro* dry matter digestibility (IVDMD) was calculated using the equation of Tilley and Terry (1963). *In vitro* neutral detergent fiber digestibility (IVNDFD) and *in vitro* acid detergent fiber digestibility (IVADFD) were calculated using the equation described by Cherney et al. (2004).

Statistical analysis

All data, except *in vitro* were subjected for analysis of variance (ANOVA) using Proc GLM. When a significant *F* - test was declared at $P < 0.05$, the least significant different test (LSD) option of SAS (1989) was used for treatment mean separation. Orthogonal polynomials were used to analyze the linear and quadratic response using SAS (1989). Data from *in vitro* digestion were subjected for analysis of variance (ANOVA) using Proc ANOVA. When a significant *F* - test was declared at $P < 0.05$, the Duncan's multiple range tests (DMRT) option of SAS (1989) was used for treatment mean separation.

Table 1 Ingredients and chemical compositions of experimental diets

Item[1]	Control	OY4	OY8	OY12	PO3
Ingredient, % of DM					
Napier silage	40.0	40.0	40.0	40.0	40.0
Cassava chip	36.5	34.0	32.0	31.0	33.3
Soybean meal	15.5	14.5	13.6	13.0	15.7
Palm kernel meal	5.0	4.5	3.4	1.0	5.0
Oleaginous yeast	-	4.0	8.0	12.0	-
Palm oil	-	-	-	-	3.0
Molasses	2.0	2.0	2.0	2.0	2.0
Urea	0.5	0.5	0.5	0.5	0.5
Mineral - vitamin mix[2]	0.5	0.5	0.5	0.5	0.5
Composition, % of DM					
TDN[3]	70.9	71.1	71.4	71.8	73.9
CP	12.7	13.1	13.5	13.1	13.0
EE	1.9	2.7	3.9	4.7	4.7
NDF	42.9	42.4	42.6	42.7	41.5
ADF	28.2	28.1	28.8	28.7	28.4

[1]**Control** = TMR with no supplemented fat, **OY4** = TMR supplemented with 4% of oleaginous yeast, **OY8** = TMR supplemented with 8% of oleaginous yeast, **OY12** = TMR supplemented with 12% of oleaginous yeast and TMR supplemented with 3.0 % of palm oil.

2Mineral - vitamin mix contains 2,400,000 IU of vitamin A, 400,000 IU of Vitamin D, 3,000 IU of Vitamin E, 2.0 mg of Vitamin B_{12}, 10 g of Magnesium, 8.5 g of Manganese, 10 g of Iron, 10 g of Zinc, 2.0 g of Copper, 300 mg of Cobalt, 500 mg of Iodine, 40 mg of Selenium, 20.0 g of Calcium, and 10.76 g of Phosphorous.

[3]Calculated TDN

Results and discussion

Effect of carbon and nitrogen concentration on lipid production

In the present study, concentration of molasses and $(NH_4)_2SO_4$ in culture medium were optimized in order to improve lipid accumulation in *T. asahii* GSY10 when using molasses as carbon source and $(NH_4)_2SO_4$ as nitrogen source. The result showed that increasing molasses concentration from 80 to 160 g/l resulted in linear increasing in biomass and lipid yield ($P < 0.05$). The lipid yield was increased by 23.3% when increasing molasses concentration from 80 to 160 g/l. However, there was no significant difference between 120 and 160 g/l molasses on lipid yield ($P > 0.05$) (**Table 2**). In term of nitrogen, decreasing $(NH_4)_2SO_4$ concentration resulted in linear increasing in lipid yield and lipid content ($P < 0.05$). The lipid yield and lipid content were enhanced by 1.9 and 2.3 times when decreasing $(NH_4)_2SO_4$ concentration from 1.5 to 0.5 g/l ($P < 0.05$) as showed in **Table 2.** Similar observation was reported by Karatay and Dönmez (2010) who found that lipid accumulation of *Candida tropicalis* was increased when rising molasses concentration from 60 to 80 g/l and decreasing $(NH_4)_2SO_4$ from 1.5 to 0.5 g/l.

In lipid production, carbon to nitrogen ratio (C/N ratio) of culture medium is the major factor affecting lipid accumulation in oleaginous yeast. In the recent study, the C/N ratio of medium containing 120 and 160 g/l molasses with 0.5 g/l $(NH_4)_2SO_4$ were 24.1 and 24.8, respectively. Under this C/N ratio, *T. asahii* GSY10 showed its maximum lipid content as 31.4 and 27.9% of dry biomass, respectively **(Figure 1)**. The enhancing of lipid content when increasing the C/N ratio of the medium was also observed in *T. fermentans* (Zhu et al., 2008) and *T. capitatum* (Wu et al., 2011). Beopoulos et al. (2009) clearly reviewed that the medium with high C/N ratio increased citrate accumulation in mitochondria. Citrate was transported out of cytosol and cleavage onto acetyl - CoA, precursor of fatty acid synthesis. However, increasing molasses concentration over an optimum level could reduce dry biomass and lipid accumulation owing to the toxic effect of molasses on cell growth (Karatay and Dönmez, 2010). The slight reduction of lipid content when increasing molasses concentration from 120 to 160 g/l with 0.5 g/l $(NH_4)_2SO_4$ can be explain by the toxic effect of molasses on cell growth. From the result of this experiment, molasses concentration at 120 g/l and $(NH_4)_2SO_4$ concentration at 0.5 g/l were used for further experimentation.

Table 2 Effect of molasses and $(NH_4)_2SO_4$ concentration on lipid accumulation in *T. asahii* GSY10.

	Molasses (M), g/l			$(NH_4)_2SO_4$ (N), g/l				Effect of treatment			Effect of M[1]		Effect of N[2]	
Item	80	120	160	1.5	1.0	0.5	SEM	M	N	M*N	L	Q	L	Q
Biomass	10.30^c	11.60^b	12.97^a	12.19^a	12.27^a	10.41^b	0.72	<0.01	<0.01	0.31	<0.01	0.96	<0.01	0.07
Lipid yield	2.04^b	2.36^a	2.66^a	1.54^c	2.58^b	2.94^a	0.18	<0.01	<0.01	0.11	<0.01	0.91	<0.01	0.02
Lipid content	19.90	20.70	21.20	12.51^c	20.94^b	28.38^a	1.59	0.62	<0.01	0.14	0.34	0.90	<0.01	0.67

[1]L = linear effect of molasses concentration, and Q = quadratic effect of molasses concentration.

[2]L = linear effect of $(NH_4)_2SO_4$ concentration, and Q = quadratic effect of $(NH_4)_2SO_4$ concentration.

[a,b,c]Means within the same row are not sharing common superscripts differ significantly ($P < 0.05$).

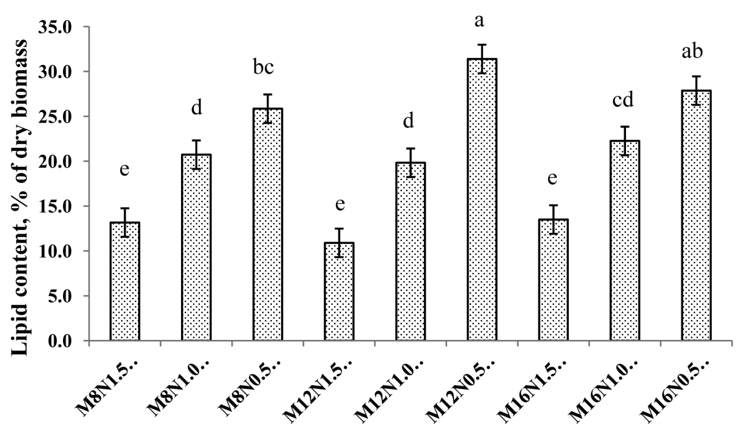

Figure 1 Effect of molasses (M; 80, 120, 160 g/l) and $(NH_4)_2SO_4$ (N; 1.5, 1.0, 0.5 g/l) concentration on lipid content of *T. asahii* GSY10. [a,b,c,d,e]Means not sharing common superscripts differ significantly ($P < 0.05$).

Effect of initial pH, cultural temperature, and cultural time on lipid accumulation

In the present study, optimum initial pH, cultural temperature, and cultural time were examined. The results revealed that dry biomass, lipid content, and lipid yield were linearly decreased when increased the initial pH from 4.0 to 6.0 **(Figure 2a)**. However, the dry biomass and lipid content showed no significant difference among pH value 4.0 and 5.0 (P > 0.05). This indicated that pH value ranging between 4.0 and 5.0 were suitable for lipid accumulation from molasses by *T. asahii* GSY10. Similar optimal pH for lipid accumulation was observed in *T. capitatum* (Wu et al., 2011). However, the optimal pH for lipid accumulation in the present study was lower than the optimal pH for lipid accumulation of *T. fermentans* (pH 6.0) when using molasses as carbon source (Zhu et al. 2008). Several reports showed that optimal pH for lipid accumulation in oleaginous yeast varied from 4.0 to 6.5 depending upon their phenotype and carbon source (Karatay and Dönmez, 2010; Angerbauer et al., 2012; Chen et al., 2013).

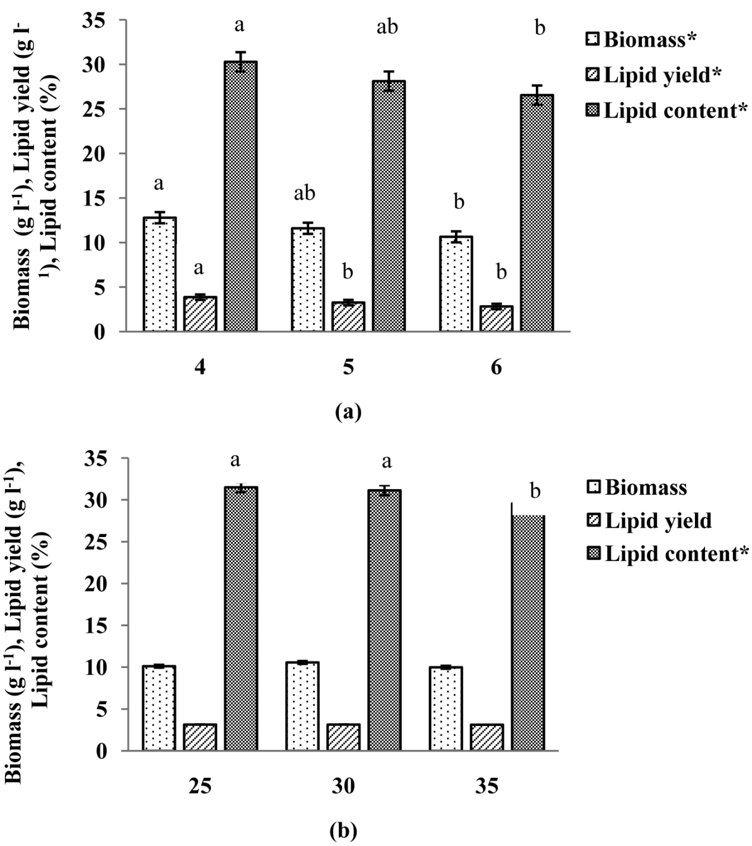

Figure 2 Effect of initial pH value (a) and cultural temperature (b) on lipid accumulation of *T. asahii* GSY10. The culture media contained 120 g/l molasses and 0.5 g/l $(NH_4)_2SO_4$. Cultures were performed at 150 rpm for 6 days. Symbol (*) showed linearly response of parameters. [a, b, c]Means are not sharing common superscripts differ significantly (P < 0.05).

In microbial lipid production, cultural temperature plays an importance role in lipid accumulation (Saxena et al., 1998). The present study illustrated that the increase of cultural temperature from 25 to 35 °C resulted in a linear decrease in lipid content ($P < 0.05$) as showed in **Figure 2b**. However, the lipid content had no significantly difference between cultural temperature at 25 and 30 °C. Further rise in cultural temperature beyond 30 °C led to 5.1% decreasing in lipid content ($P < 0.05$). This indicated that cultural temperature lower than 30 °C did not affect the lipid accumulation in *T. asahii GSY10*. Numerous researches reported that the optimum temperature for lipid accumulation in oleaginous yeast ranged from 25 to 32 °C. This depended on their type strain (Hall and Radledge, 1977; Saxena et al., 1998; Chen et al., 2013). Increasing cultural temperature over optimum temperature diminished the activity of enzymes involving in lipid biosynthesis (Saxena et al., 1998; Wu et al., 2011).

In order to examine the effect of cultural time, *T. asahii* GSY10 was cultured in molasses medium with increasing time course from 2 to 10 days. The result illustrated that maximum lipid yield and lipid content were obtained on day 8 of fermentation (4.1 g/l and 39.4% respectively). Further rise in cultural time beyond 8 days resulted in lipid yield and lipid content decreasing (Figure 3). This resulted from the breakdown of the accumulated lipid for cell proliferation due to the lack of carbon (Zhu et al., 2008; Wu et al., 2011). Holdsworth and Ratledge (1998) determined the lipid turnover in oleaginous yeast by transferring cells with storage lipid into a carbon - starvation medium. The result found that the storage lipid was rapidly utilized after the carbon exhaustion.

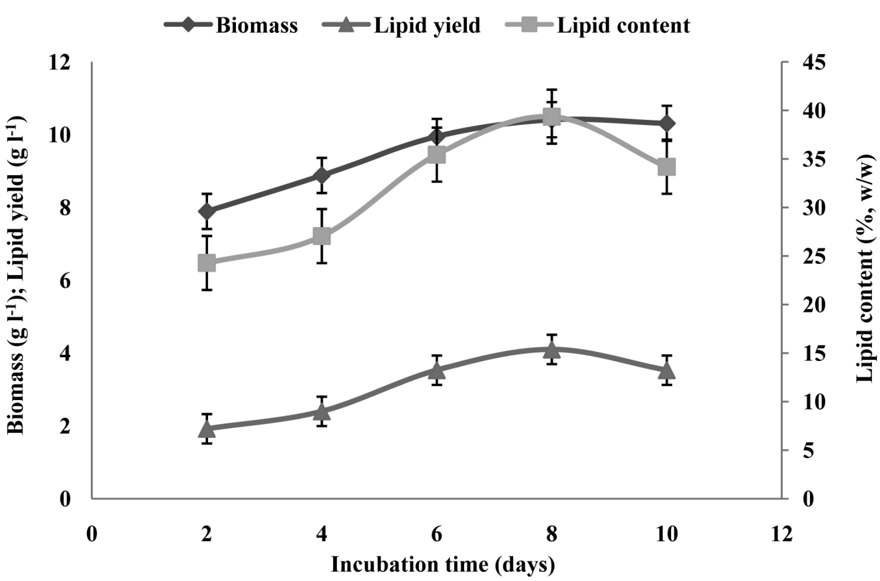

Figure 3 Effect of cultural time on lipid production by *T. asahii* GSY10. Culture media contained 120 g/l molasses, 0.5 g/l $(NH_4)_2SO_4$ with adjusted pH onto 5. Inoculums concentration was 10%. All cultures were performed at 30 °C, 150 rpm.

Effect of oleaginous yeast supplementation on *in vitro* nutrient digestion

Supplementation of *T. asahii* GSY10 at levels 4.0 to 12.0% of DM did not affect *in vitro* dry matter digestibility (IVDMD) when compared with control ($P > 0.05$). However, increasing oleaginous yeast supplementation from 8.0 to 12.0% resulted in 5.3% decreasing in IVDMD ($P < 0.05$) as showed in **Figure 4**. Whereas supplementation of palm oil markedly decreased IVDMD when compared with the other treatments ($P < 0.05$). This indicated that fat supplementation from yeast had less toxicity on ruminal digestion than free oil. Ando et al. (2004) illustrated that dried beer yeast had high difficult degradation fraction (88%) and low degradation rate (6.2%/h). Low degradation rate of yeast in the rumen might relate to slow releasing of lipid storage within the cell. Reddy et al. (1994) reported that free oil had higher fatty acids releasing rate than other forms. This fatty acids inhibited microbial growth and prevented microbial adherence to feed particles, that decreasing dry matter digestibility (Palmquist and Jenkin, 1980). Supplementation of *T. asahii* GSY10 in TMR tended to improve *in vitro* neutral detergent fiber digestibility (IVNDFD) and *in vitro* acid detergent fiber digestibility (IVADFD) as showed in **Figure 4** ($P < 0.10$). Callaway and Martin (1997) reported that yeast supplementation stimulated the growth of cellulolytic bacteria. This resulted in the increased of cellulose digestion.

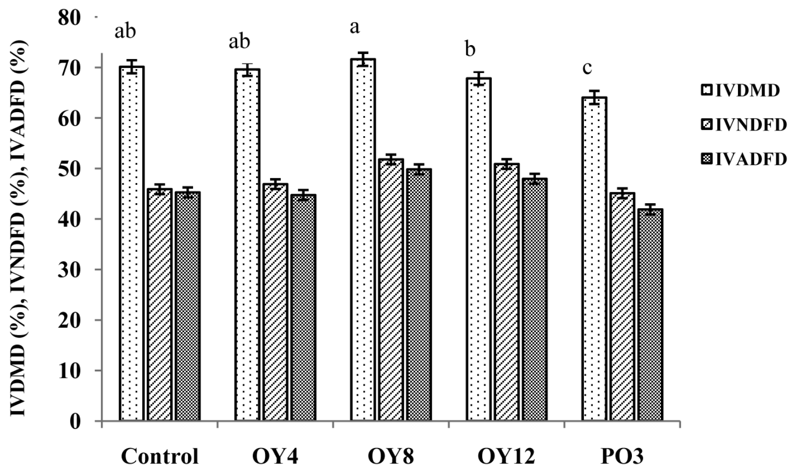

Figure 4 Effect of *T. asahii* GSY10 supplementation on *in vitro* dry matter digestibility (IVDMD), *in vitro* neutral detergent fiber digestibility (IVNDFD) and *in vitro* acid detergent fiber digestibility (IVADFD) of TMR. Control = TMR with no fat, OY4 = TMR with 4.0% DM of oleaginous yeast, OY8 = TMR with 8.0% DM of oleaginous yeast, OY12 = TMR with 12.0% DM of oleaginous yeast, and PO3 = TMR with 3.0% DM of palm oil.

Conclusion

Lipid accumulation from molasses in *T. asahii* GSY10 was improved by increasing molasses concentration from 80 to 120 or 160 g/l and reducing $(NH_4)_2SO_4$ concentration in cultural media from 1.5 to 0.5 g/l. Cultivation of *T. asahii* GSY10 in nitrogen - limited media containing 120 g/l molasses, 0.5 g/l $(NH_4)_2SO_4$, with adjusted pH onto 5, and incubated at 30°C for 8 days, dry biomass, lipid yield, and lipid content reached to 10.4 g/l, 4.1 g/l, and 39.4% of dry biomass, respectively. The lipid content was improved up to 67.7% when compared with original value (39.4% vs 23.5%). Supplementation of oleaginous yeast, *T. asahii* GSY10 in TMR did not affect IVDMD, IVNDFD, and IVADFD. However, 8% of its supplementation tended to improve fiber digestion ($P < 0.1$). Palm oil supplementation at level of 3% DM depressed dry matter and fiber digestibility. Oleaginous yeast has a potential to use as alternative fat source in ruminant diet.

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