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IN VITROCONJUGATED LINOLEIC ACID FORMATION BY NEWLY ISOLATED ANAEROBIC GUT FUNGI

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Abstract

Anaerobic gut fungi (AGF) contribute to the production of conjugated linoleic acid (CLA)which is a collective mixture of isomers (28 different isomers)mainly from linoleic acid (LA) and essential fatty acids. Of these isomers,c-9, t-11 and trans-10, cis-12 isomers are found to have physiological effects on human'shealth while rumenic acid can be found within 72 - 94 % of overall CLA in the ruminal food products. Here, the CLA production of6fungalisolates (three Orpinomyces sp. and threeNeocallimastix sp.) when they were incubated in Orpin's medium lacking energy source and in the presence of 1 mg/ml of Linoleic Acid in the medium was investigated. All the culture media were incubated at 39°C for the time intervals of 0, 2, 4, 6, 8 and 10 hrs and tubes put into ice after incubation completed to stop any further reactions. Produced CLA in the medium was extracted and the amount of the conversion from linoleic acid (LA) to CLA was determined using UV spectrophotometer (233nm).This study found that comparing to each other, Orpinomyces sp. are more likely to be involved in CLA production than Neocallimastix sp. In another hand, there are variation in CLA production even between strains of same species as we found in this study that one strain of Orpinomyces sp. has higher ability in CLA production than the two others. It should also be noted according to the results of this study that using linoleic acid as a growth substrate in the medium instead of glucose or cellobiose, enhances the production of CLA and shortens the time needed for the process.

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Keywords: Rumen, anaerobic fungi, CLA, gut microbiology

I. INTRODUCTION

Anaerobic gut fungi (AGF) are one of the strictly anaerobic microorganism inhabit mainly in the rumen of ruminants and caecum of single stomached herbivores, although they are distributed in different parts of the gastro intestinal tract (GIT) (Davies et al., 1997). They are considered as a key player in helping ruminants for biodegradation of the lignocellulosic plant fibers ingested by host herbivores (Comlekcioglu et al., 2010; Macouzet, Lee, and Robert 2010). Lignocellulosic material is degraded by anaerobic fungi as their rhizoids are grown on the surface of plant biomass by penetrating them with the using of variety of powerful polysaccharide-degrading enzymes. Recent study has showed valuable information about the diversity of these enzymes and their activity in degrading carbohydrate within *Orpinomyces sp.* and the gene responsible for such enzymes has been horizontally acquired from the rumen bacteria (Youssef et al. 2013). Numerous advantages validated from the presence of anaerobic fungi within the rumen that have resulted in growing interest of their use as a pre/probiotic over the last decade (Ozkose et al., 2009).Although their basic role is the biodegradation of fibrolytic feed stuff in the rumen they have some other pivotal functions which are, hitherto, relatively little studied. AGF contribute to the production of CLA naturally, for instance, by fermentation and dehydrogenation of linoleic acid into CLA and trans-11-18:1 (vaccenic acid) which is the predominant trans monounsaturated fatty acid of animal tissue fat (Bhattacharya et al. 2006).

Conjugated linoleic acid defined as a family of 28 isomers from linoleic acid and an essential fatty acid. Sofar more than 28 different isomers of the CLA were reported, and although most of these isomers show biological activity, only two of them (namely cis-9, trans-11 isomer which is also called as rumenic acid and trans-10, cis-12 isomer)are known to have physiological effects on humans health(Bhattacharya et al. 2006; Churruca, et al. 2009). Rumenic acid contributes to large portion of foods with the ratio ranges between 72 to 94% of total CLA in foods, while t10, c12 isomer of the CLA is found relatively in lesser amount of milk and meat products of ruminant animals (Ing and Belury, 2011; Jutzeler van Wijlen 2011) . CLA formation in rumen mainly related to the microorganisms that have the ability to release lipases enzyme.- GIT of mammalian herbivores harbour various anaerobic microorganisms (i.e. fungi, bacteria, archaea, and protozoa) which produce lipase enzymes for the biodegradation of lipids derived from plant biomass ingested by animals (Belanche et al. 2012). These microorganisms have a symbiotic relationship with their natural host herbivorous mammalians. There are extensive studies going on the GIT ecosystems of these animals, since microorganisms present in gut system play a notable role in the human nutrition and health (Chaucheyras-Durand and Ossa 2014).There are increasing evidences that human chronic diseases including cancer, cardiovascular disease, insulin resistance and obesity can be altered or reversed by the nutrition habits.

Few reports confirmed the ability of different AGF for the production of CLA and to obtain optimum conditions for bio dehydrogenation of LA to CLA or VA, the rumen fungi medium should lack the soluble carbohydrates, VFA or vitamins and the pH should be set to 6.5(I. S. Nam and Garnsworthy 2007a,b; Çömlekcioğlu et al. 2011).Although several reports published about CLA production by ruminal bacteria and lactic acid bacteria (Bauman et al. 1999; Lourenço,

Ramos-Morales, and Wallace 2010; Gorissen et al. 2013a; Kuhl and Lindner 2016), the current knowledge about the role of AGF on the CLA formation is quite limited yet.

In this study, therefore, possible roles of six fungal isolates (purified from freshly voided faecal samples and putatively identified as *Neocallimastix*spp. (n=3) and *Orpinomyces* spp. (n=3)) for the CLA production were investigated. Furthermore, CLA formation capability of these fungal isolates was discussed when they were grown in Orpin's medium containing linoleic acid as sole energy source.

II. MATERIAL AND METHOD

Microogranisms and isolation process

Two anaerobic fungal isolates *Neocallimastix* sp. GMLF23 and *Orpinomyces* sp. GMLF5, obtained from the culture collection of Biotechnology and Gene Engineering Laboratory of Animal Science department, Agriculture Faculty at Kahramanmaras Sutcu Imam University. Four isolates of two genera of AGF, namely *Neocallimastix* and *Orpinomyces*, were isolatedfrom freshly produced goat faeces and purified using role tube technique of Joblin(1981). Following putative characterization using morphological features, these newly purified isolates are designated as *Neocallimastix* GMLF150 and *Neocallimastix* GMLF151, *Orpinomyces* GMLF156 and *Orpinomyces*GMLF157.

Media and culture conditions

The anaerobic nutrient medium for AGF was based on the basal medium of Hobson (1969), which was adapted by Orpin (1977) including 15% (v/v) rumen fluid. Fresh rumen content collected from the slaughterhouse and immediately brought to the laboratory where it was strained through four layers of cheeseclothto remove plant biomass. The liquor was, then, centrifuged at 18000 *g* for 30 min, clarified liquor is either used freshly or kept in -20 ^{o}C until further usage. The medium was prepared by adding 10 ml of basal medium into Hungate tubes containing 5 mg ml^{-1} (final concentration) energy source. To inhibit bacterial growth, 25 and 50 mg ml^{-1} final concentrations of mixture of streptomycin sulfate and choloramphenicol(both prepared in 50% EtOH) were used respectively. All fungal isolates were maintained at 39^{o}C and subculture by transfer of 10% to fresh medium twice in a week throughout the experiments. The growth of fungal isolates were observed using Olympus CK-2 inverted light microscope with bright field illumination.

LA stock solution

LA stock solution prepared according to the report of Kim and Liu (2000) with slight modification. Concentrated LA (0.902 g/ml, Sigma-Aldrich) dissolved in de ionized water containing tween 80 (1% v/v) to give the final concentration of 10 mgml^{-1}. The stock solution was vortexed and stored in -20°C until further usage.

Determination of CLA

Fungal isolates were incubated at 39 ^{0}C for 72 h and 1 ml of medium containing fungal mass was transferred into the 9 mlof anaerobicmedium without energy sources (such as straw, glucose

orcellobiose) with continuous CO_2flow it was inoculated with 1 ml of LA stock solution giving the final concentration of 1 mgml^{-1} in Hungate tubes.. The culture then kept at 39^{o}C for 0, 2, 4, 6, 8, 10 hours. Followingeach incubation period, the Hungate tubes were moved into ice immediately to stop the reaction(I. S. Nam and Garnsworthy 2007b).

Extraction of CLA

CLA extraction performed with cultures (typically 1ml) added in a 10-ml plastic test tubes containing a mixture of 3 ml organic solvents (mixture of 1-part hexane and2 parts isopropanol). The mixture centrifuged 5700 *g*(Hettich, Germany) for 5 min and the upper layers are carefully collected and 240 µg of collected solution was transferred into micro plate wells. The ODs of each well were recorded with the aid of a UV spectrophotometer (Spectramax Plus 384, UK)at 233 nm wavelength.

CLA standards

CLA standards are prepared using a dilution for purified CLA (Sigma Aldrich) according to the method described byZhao and Li (2011). Purified linoleic acid, conjugated methyl ester (Sigma), has been used to create the calibration curve. Hexane was used to dilute the CLA serially and the curve has been produced (R^2=0.99) by reading the absorbance at 233 nm wavelength. Calibration curve produced between the concentration of purified CLA and their optical density inside the hexane. For positive strains, the phenotypic screening experiments were performed in triplicate (two tubes for each replication) and conversion percentages were represented as means ± standard deviations.

III. RESULTS

Detection of CLA production by *Orpinomyces* spp

All three isolates of*Opinomyces*(GMLF5, GMLF156and GMLF157)were treated with 10 µg/ml of linoleic acid solution. Following the incubation at 39°C for 0, 2, 4, 6, 8, 10 hours, the serum bottle kept in ice to stop the reaction. The produced CLA inside each tube has been extracted and their optical density has been read at 233 nm using UV spectrophotometer.

Figure 1, demonstrates CLA formation rate for*Orpinomyces* sp. GMLF5 at different time intervals. For zero time of incubation, the CLA recorded as 30 µg/ml, then with increase in incubation period for up to 2 hours this rate was increased into 34 µg/ml. The CLA production recorded as 43 and 47 µg/ml when the incubation period increased to 4 and 6 hours respectively. CLA production rate was low with maximum production rate of 49 µg/ml recorded after 8 hours of incubation.

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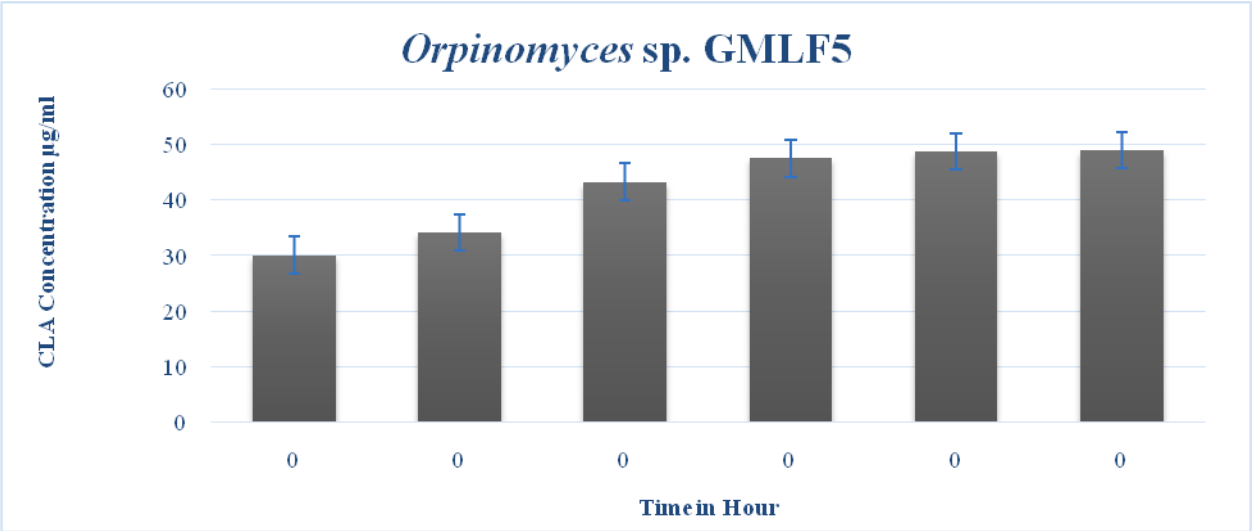

Figure 1. CLA concentration within time intervals produced by *Orpinomyces* sp. GMLF5. Error bars are representing the standard error of mean (n=6).

Newly isolated and purified *Orpinomyces* sp. GMLF156 were subjected to CLA production as well (Figure 2). The CLA concentration was calculated as 22µg/ml in the zero time. This rate increased when further incubation applied for 2 hours at 39 °C to become 26 µg/ml reflecting the OD of 1.143. The CLA production rate continued to increase with further incubation reaching to 37 and 42 µg/ml reflecting the OD read as 1.347 and 1.441 for the incubation periods of 4 and 6 hours respectively. The highest CLA rate produced by this strain were recorded at the incubation time of 8 hours which were 43 µg/ml then declined remarkably to 39 µg/ml when further incubated to 10 hours.

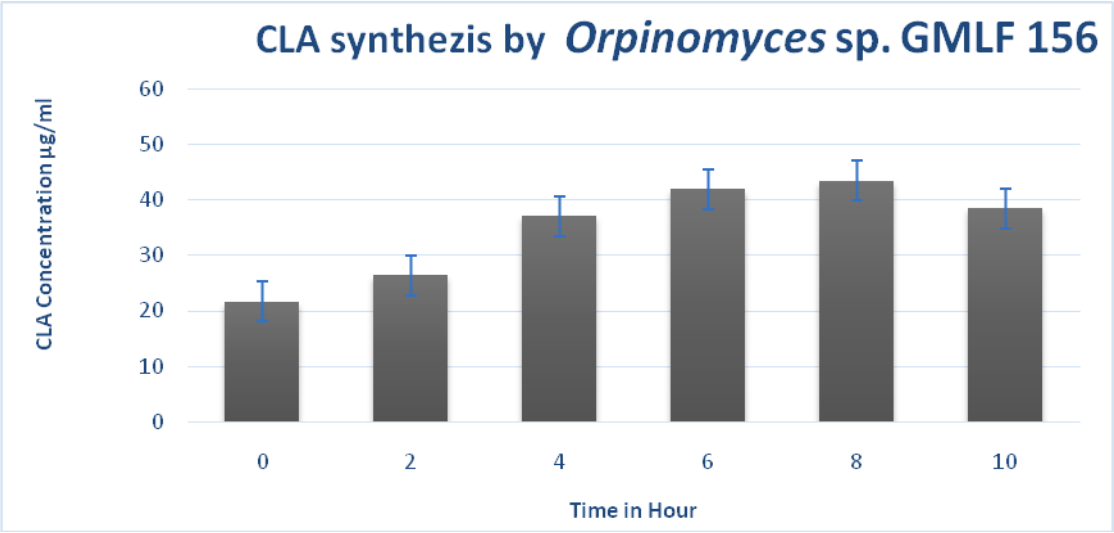

Figure 1. CLA concentration within time intervals produced by *Orpinomyces* sp. GMLF156.Error bars are representing the standard error of mean (n=6).

Another newly isolated polycentric *Orpinomyces* sp. GMLF157 were also subjected to CLA production and it showed the highest activity comparing to the other two isolates (GMLF155 and

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GMLF15) of this genus (Figure 3). The CLA concentration were 55 µg/ml in the zero time of incubation at 39 ^{0}C. This rate increased sharply to reach 2,323 µg/ml when it was further incubated at 39 ± 1°C for two hours, reflecting the OD read as 2.929. Then the CLA production continued to increase into 3,065 µg/ml with OD read as 2.966 when the isolate incubation time increased in to four hours. Further increase in incubation time to six hours the CLA production rate increased in to 3,305 µg/ml with OD read as 2.978. Its sharply decreased in 8thhour ofincubation in a negligible amount (7 µg/ml).

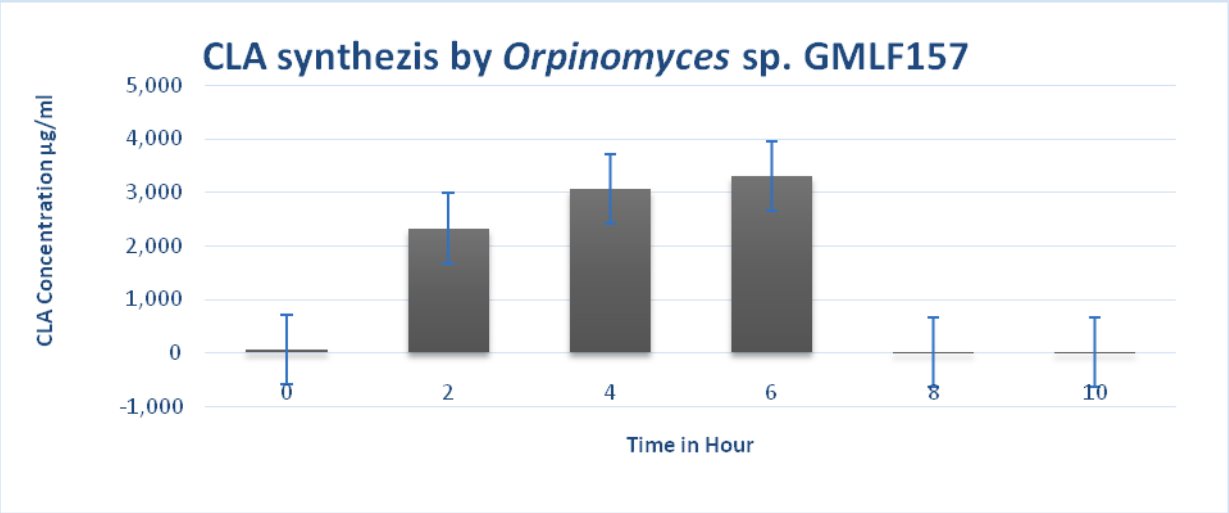

Figure 2. CLA concentration within time intervals of 0 to 10 hours produced by *Orpinomyces* sp.GMLF157. Error bars are representing the standard error of mean (n=6).

Detection of CLA production by *Neocallimastix* spp

Two isolates (designated as GMLF150 and GMLF151 and deposited in our culture collection) were purified and putatively characterized as *Neocallimastix* spp. These two newly purified isolatesand*Neocallimastix*spGMLF123 (obtained from our culture collection)have been treated with 10 µg/ml of linoleic acid solution. Then after incubation at 39 ^{o}C (for 0, 2, 4, 6, 8, 10hours), the serum bottles kept in ice to stop the reaction immediately. The produced CLA inside each tube has been extracted and their optical density has been read with the aid of spectrophotometer.

Figure4, demonstrated that newly isolated*Neocallimastix sp*.GMLF150was able to produce CLA at very low concentration and the highest CLA production rate was recorded at 6 hours incubation responding to 45µg/ml.

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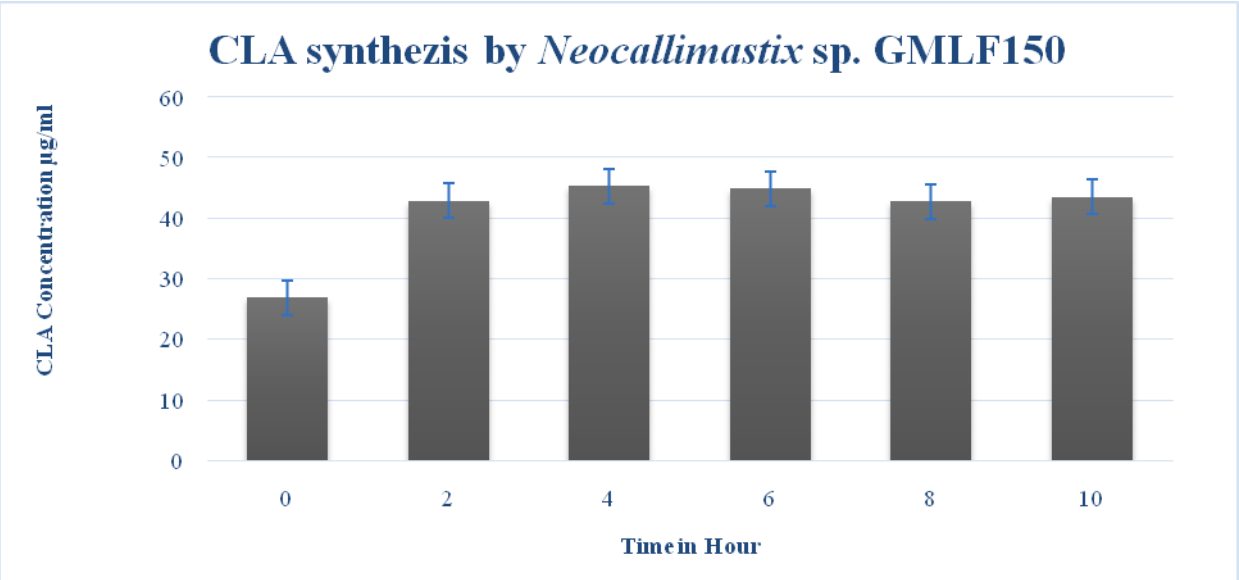

Figure 3. CLA concentration within time intervals produced by *Neocallimastix* sp.GMLF150.Error bars are representing the standard error of mean (n=6).

Newly isolated and purified *Neocallimastix* sp. GMLF 151 isolate was able to produce CLA at low concentration and the highest CLA production rate was recorded at 6 hours incubation responding to 42 µg/ml (Figure 5).

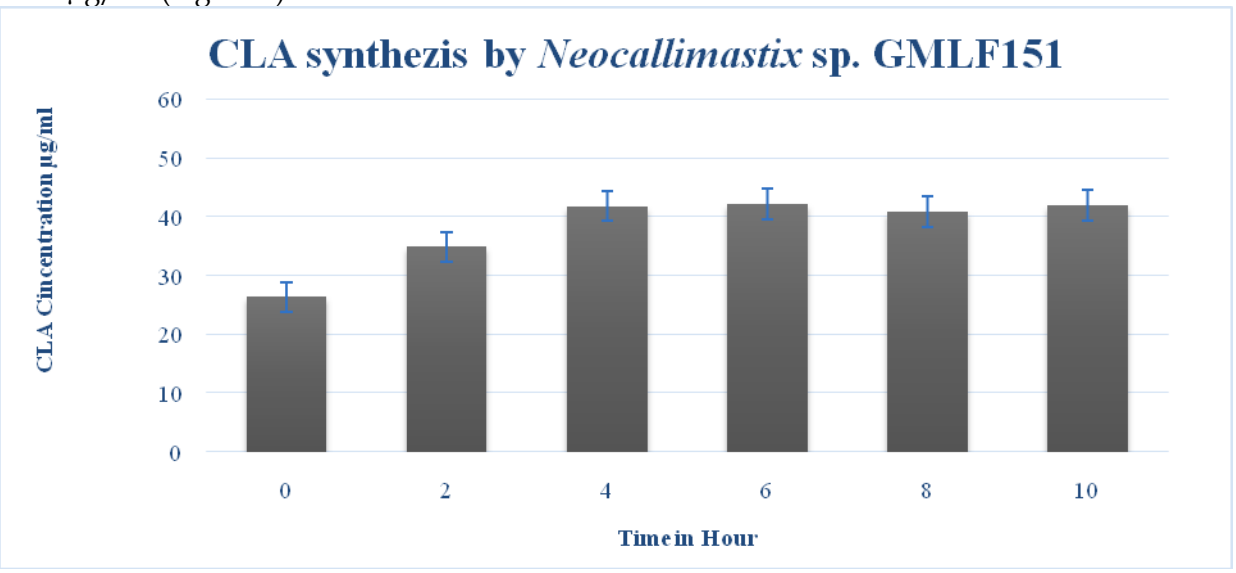

Figure 4. CLA centration within time intervals produced by *Neocallimastix* sp.GMLF151. Error bars are representing the standard error of mean (n=6).

In figure 6, CLA production within the *Neocallimastix* sp.GMLF23were recorded as 33 µg/ml within the incubation time of zero. Then this rate can be seen increased into 34 µg/ml with the increase of incubation time up to 2 hours. This production rate continued into 43, 47 and 49 when the incubation period increased into 4, 6 and 8 hours respectively. The rate remained constant with

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increase in incubation period for up to 10 hours.

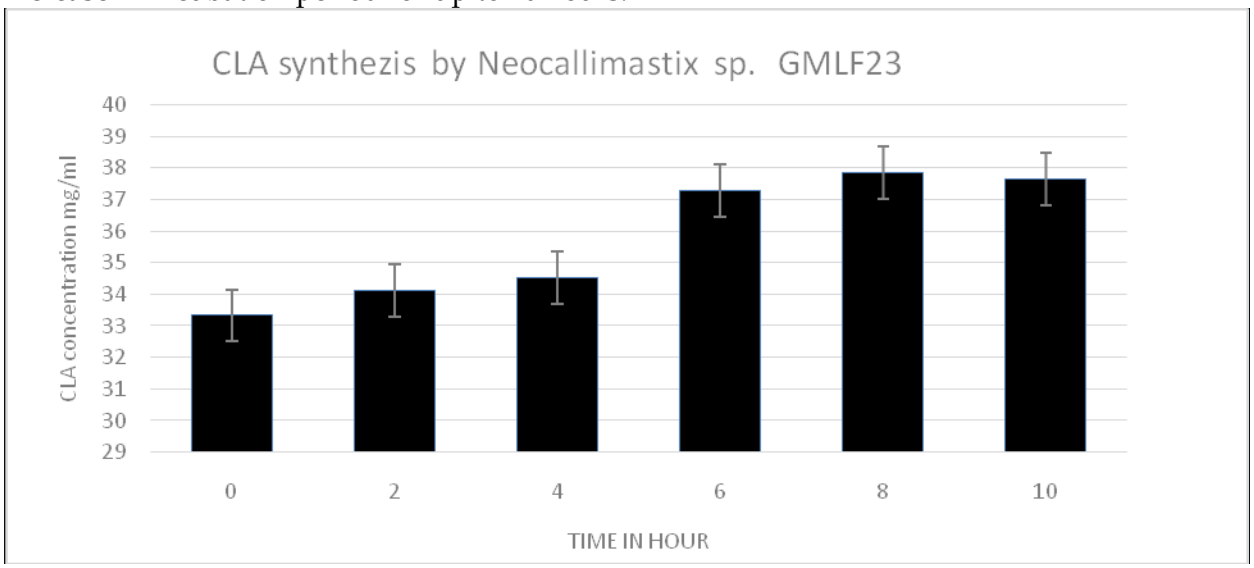

Figure 6. CLA concentration within time intervals produced by *Neocallimastix* sp.GMLF123.Error bars are representing the standard error of mean (n=6).

IV. DISCUSSION

Biohydrogenation of linoleic acid to conjugated linoleic acid by different isolates of *Orpinomyces*spp and *Neocallimastix*spp has been determined according to previous work done by (I. S. Nam and Garnsworthy 2007b) with slight modification. In this study, when faecal samples from goats cultured an aerobically, two different isolates of *Orpinomyces* sp. with two different isolates of *Neocallimastix* sp. has been purified.

(In Sik Nam and Garnsworthy 2006) has reported that the bio hydrogenation of linoleic acid to vaccenic acid completely took 24 hrs, when mixed rumen fungi used. However, our study found that when only linoleic acid is added to Orpin's medium as an energy source, the biohydrogenation of linoleic acid to Vaccenic Acid accelerates thus the appearance of the CLA as a medium product are quicker. When pure culture of different *Orpinomyces* sp. has been inoculated with 10 µg/ml of LA solution, then after incubation at 39 °C for 0, 2, 4, 6, 8, 10 hours, it was observed from three strains, that, two have notably high capability to dehydrogenate linoleic acid and production of CLA started within the first hours of the incubation. The highest proportion of CLA produced by one of the *Orpinomyces sp.* was 3,305 µg/ml after 6 hours incubation. While same species showed sharp decrease in the production at 8 and10 hours incubation, this means that the fungi has stopped biohydrogenation of Linoleic acid after 6 hours. Furthermore, one of the strains showed very low activity in biohydrogenation of the linoleic acid and production of CLA, which only 42 µg/ml after six hours of incubation. This result shows that some strains within same species have different capability in production of CLA. *Neocallimastix* sp. also, has been purified and subjected to growth in the presence of 1 mg/ml of Linoleic Acid solution. Then after the incubation of two strains showed that they have capability in biohydrogenation of LA but

relatively lesser amount, with the highest concentration of CLA produced 45 µg/ml within 6 hours incubation.

Anaerobic fungal strains were grown in anaerobic medium containing linoleic acid (1mg/ml) as an energy source although usage of the wheat straw, cellulose, cellobiose, xylan etc is a common application for the culturing of anaerobic fungi (Orpin, 1976; Griffith et al., 2009; Hanafy et al., 2017) at the concentrations of 2-5 g/L. The CLA production was recorded up to 8 hours incubation within some strains. These resultsare in agreement with previous study reported by(In Sik Nam and Garnsworthy 2006) indicating that supplementing glucose or cellobiose to the Orpins medium leads to inhibition in the biohydrogenation ofLA by fungi compared to the using linoleic acid solely as supplement. There are many reasons that might be interfere with the biohydrogenation capacity, like the end product of the cellobiose fermentation are lactate, acetate, hydrogen and carbon dioxide, the accumulation of one of them might prevent the process, or during the zoospore formation, there might be a reduction in biohydrogenation ability.

Previous studies showed that pH and temperature are effecting the conversion rate of Linoleic acid to CLA for lactic acid bacteria (Martin and Jenkins 2002; Gorissen et al. 2013b) . While in thisstudy the pH were adjusted in the medium to 7.0±0.2 , which is the optimal pH range for the rumen fungi to convert LA into CLA based on previous study (Lee 2013), but the temperature remained at 39^{o}C, due to the optimal temperature for rumen fungi to grow. Conversion percentage of the CLA were calculated based on Purified Linoleic acid, Conjugated methyl ester (Sigma), which has been used to create the calibration curve.

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