

Review Article

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Current Knowledge on Source and Synthesis of Conjugated Linoleic Acid (CLA): A Review



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Abstract

Dietary fatty acids are highly recognized as an important biologic controller and having potential to provide number of health benefits. Conjugated linoleic acid is an important conjugated fatty acid with a mixture of positional and geometric isomers of linoleic acid (C18:2, n-6). In the last three decades, this particular type of PUFA has gained significant attention for their biologically active properties and health effects. CLA can be synthesized endogenously in tissues and partial bio hydrogenation of unsaturated fatty acids in rumen stomach. Also, it can be synthesized by using microbial strains like *Lactobacillus* spp and *Butyrivibrio fibrislovensis* but the amount of yield obtained is not satisfactory. Application of homogenous and heterogeneous catalysts and photoisomerization methods increased the conversion of linoleic acid to CLA. In 2008, CLA got generally recognized as safe (GRAS) status by FDA, which increased the consumption of CLA and used as a nutraceutical in dietary supplements/foods. With the increasing market demand, the research should focus in developing innovative synthesis methods with enhanced productivity of specific CLA isomers. The main scope of this review is to outline the current knowledge on different types of synthesis of CLA isomers and sources rich in CLA.

Keywords: Conjugated linoleic acid; Sources; Biosynthesis; Catalysts; Enzymes

Introduction

Lipids may be bounded as “fatty acids, their derivatives, and substances connected biosynthetically or functionally to these compounds” [1]. It mainly consists of triacylglycerol (TAG’s), which are molecules composed of three fatty acids and glycerol (phosphatidylcholine and cholesterol also included) [2] and generally found in the tissues of microorganisms, plants, animals and insects [3-6]. TAG’s and fatty acids accelerated the absorption of other fat-soluble components such as vitamins. As well with proteins, carbohydrates, and alcohol, fats are a major source of energy for the body and also serve many essential functions (e.g. Structural components of cell membrane, regulators of enzyme activities (e.g. protein myristoylation), precursors for bioactive molecules, and regulation of gene expression). Fatty acids can be categorized according to their number of double bonds. Saturated fatty acids (SFA) have no double bonds, while monounsaturated fatty acids (MUFA) have one double bond and polyunsaturated fatty acids (PUFA) have two or more double bonds [2]. PUFA have 2 to 6 double bonds and PUFA having 20 or more carbon atoms are usually termed as LCPUFA both are essential for physiological activities of the organism.

In specific, polyunsaturated fatty acids (PUFA’s), such as linoleic acid (18:2, n-6), α -linolenic acid (LNA, 18:3, n-3), arachidonic acid (ARA, 20:4, n-6), Eicosapentenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3) are termed

as conjugated fatty acids (CFA’s). Conjugated fatty acids are the positional and geometric isomers of unsaturated fatty acids, containing one or more non-methylene contributed double bonds in either cis or trans conformation (Figure 1).

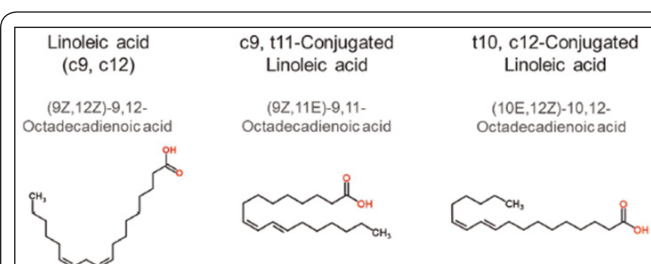


Figure 1: Structure of linoleic acid, cis-9 trans trans-11 CLA isomer and trans-10 cis-12 CLA isomer (Adapted from Viladomiu et al. [9]).

The first bioactivity of conjugated fatty acid was reported in 1979, an anti-carcinogenic component in ground beef extract which reduced the tumors in the mice which was strongly exposed to a carcinogen. This unique anti-carcinogen was not doped-out until almost a decade later, in 1987, Michael Pariza, the scientist who ascertained conjugated linoleic acid (CLA), later commented that few ant carcinogens, and probably no other known fatty acids, are as effective as CLA in preventing carcinogenesis [7,8]. Since then, CLA isomers have been

connected with a range of bioactivities including anti-microbial, anti-diabetogenic, anti-atherosclerotic, anti-obesogenic, along with enhanced bone formation, increased immune functions and improved body fat metabolism [9-15].

CLA can be found in dairy foods, meat from ruminants and some vegetable oils. Naturally CLA will occur in tissues by endogenous conversion of trans-vaccenic acid and partial biohydrogenation of unsaturated fatty acid in rumen stomach. Microbial synthesis using bacterial strains also possible but the rate of conversion and yield is not satisfactory. Chemical synthesis using metal catalysts and photoisomerization methods provided better conversion rate and yield. With GRAS status by FDA in 2008 and containing number of health giving properties, CLA can be used in certain food categories like yogurt, meal replacement shakes, fluid milk, fruit juices, nutritional bars and soy milk. The primary focus of this paper is to review the current knowledge about the synthesis of CLA isomers and sources rich in CLA.

Sources of CLA

Food products obtained from ruminant animals are the principal resources of CLA in the human diet. In dairy products, CLA content ranges from 2.9-8.92mg CLA/g fat, of which the 73-93 percent of total CLA is c-9, t-11 CLA isomer [16]. The CLA content in cheeses typically ranges from 3.59 to 7.96mg CLA/g fat. Cheeses like Blue, brie, edam and Swiss contain higher CLA content than other cheeses particularly in sharp cheddar cheese the concentration of CLA is higher than medium cheddar cheese [17,18].

In cultured dairy products, CLA content ranges from 3.82 to 4.66mg CLA/g fat. CLA content of cow's milk typically ranges from 3.38 to 6.39mg CLA/g fat; whereas, significant variation of CLA content of cow's milk products occurs [19]. These variations mainly attributed to individuals and herds and between seasons. Animals grazing in sunlight enhance the CLA concentration in milk. Anyhow, human milk always tends to contain low level of CLA than cow milk ranges from 0.37% to 0.75% of fat [20]. The major CLA isomer in bovine [21] and human [20] milk was reported to be the c-9, t-11 CLA isomer.

Human diet contains meat and meat products which is obtained from variety of animal species (Table 1). Particularly meat from grass-fed animals has greater concentration of CLA than meat from non grass-fed animals. Beef contains low level of CLA (1.2-10.0mg/g lipid) than lamb (4.3-19.0mg/g lipid) and turkey (2-2.5mg/g lipid). As well as meat from poultry, pork and horse meat shown only 1mg/g lipid [22,23]. CLA level in meat that is less common in human diet like meat from elk (1.3-2.1mg CLA per gram fatty acid methyl ester (FAME)), water buffalo (1.83mg/g fatty acids), zebu-type cattle (1.47mg/g fatty acids) and bison (2.9-4.8mg/g FAME). Adipose tissue of kangaroos contains higher CLA concentration (38mg/g fatty acids) than all animals [24].

Table 1: Range of CLA content in meat products (in mg/g FAME) [22,23].

Meat Product (Mg /G Fame)	CLA Content
Salami	4.2
Knackwurst	3.7
Black Pudding	3.0
Mortadella	2.9
Wiener	1.5/3.6
Liver Sausage	3.3
Cooked Ham	2.7
Beef Frank	3.3
Turkey Frank	1.6
Beef Smoked Sausage	3.8
Smoked Bacon	0.8-2.6
Smoked Bratwurst	2.4
Smoked German Sausage	4.4
Smoked Ham	2.9
Smoked Turkey	2.4
Minced Meat	3.5
Corned Beef	6.6
Potted Meat	3.0

Final CLA content may vary in foods. Standard and comparable levels of CLA in a variety of foods are outlined in the following Table 2. Concentrations are found to be higher in food obtained from beef, lamb and dairy products than seafood, pork, poultry and vegetable oils. Current measures of usual or actual dietary intakes of CLA are strictly limited. Reports based on intake data from USDA's Continuing Survey of Food Intake of Individuals (CSFII 1994-1996) indicate that beef users consume 221mg of CLA/day; while non-beef users consume 102mg/day. Studies have pointed that high levels of CLA in the diet may strictly prevent many life-style associated diseases.

Table 2: CLA content in uncooked foods [18,22].

Food	Total CLA (Mg/G Fat)	C9, T11-Isomer (%)
Meat		
Fresh ground beef	4.3	85
Beef round	2.9	79
Beef frank	3.3	83
Beef smoked sausage	3.8	84
Veal	2.7	84
Lamb	5.6	92
Pork	0.6	82
Poultry		
Chicken	0.9	84
Fresh ground turkey	2.5	76

Seafood		
Salmon	0.3	n.d
Lake trout	0.5	n.d
Shrimp	0.6	n.d
Dairy Products		
Homogenized milk	5.5	92
Butter	4.7	88
Sour cream	4.6	90
Plain yogurt	4.8	84
Ice cream	3.6	86
Sharp cheddar cheese	3.6	93
Mozzarella cheese	4.9	95
Colby cheese	6.1	92
Cottage cheese	4.5	83
Reduced fat swiss	6.7	90
Am.processed cheese	5	93
Cheez.whiz	5	92
Vegetable Oils		
Safflower	0.7	44
Sunflower	0.4	38
Canola	0.5	44
Corn	0.2	39

Synthesis of conjugated linoleic acid isomers

The two conjugated linoleic isomers cis-9, trans-11 and trans-10, cis-12 is known to contain number of biological activities. In last three decades, research on CLA has increased, particularly in food science and medicine which was believed that some specific isomers of CLA have important bioactive properties as authenticated in different experimental designs with mice, rats or pigs [25]. Considering this, many researchers have tried to scale-up the production of CLA [26,27]. CLA can be synthesized naturally by biohydrogenation of PUFA in the rumen or microbial synthesis by use of bacterial enzymes/anaerobic bacterium and other bacterial strains or chemical synthesis which is widely employed in industries for bulk production of CLA.

Biosynthesis of CLA: Naturally CLA synthesizes endogenously in tissues/bacterial isomeriation or/and partial biohydrogenation of PUFA's from diet in the rumen adipose tissue and mammary gland [28]. In rumen, the dietary material enters a large fermentation vat; lipids in the diet are altered by microbial population present in the rumen stomach which differentiates the fatty acid profile of lipids in the diet (mostly USFA) and lipids leaving the rumen (mostly SFA) [29]. Microbes present in the rumen would transform lipids entering the rumen via two major processes called biolysis and bio hydrogenation.

Biolysis is the process of breakdown of ester linkages that connects fatty acids. The microbial lipases hydrolyze the

ester linkages in complex lipids, causing the release of fatty acids [30]. After biolysis, microbes in rumen will initiate the biohydrogenation of USFA's. Here the conversion of USFA to SFA via isomeriation to trans fatty acid intermediates, followed by hydrogenation of the double bonds [31]. The amount of fat delivered in the rumen [32] and luminal pH [33] are always considered an important factor for enhanced rate of biolysis and biohydrogenation reaction.

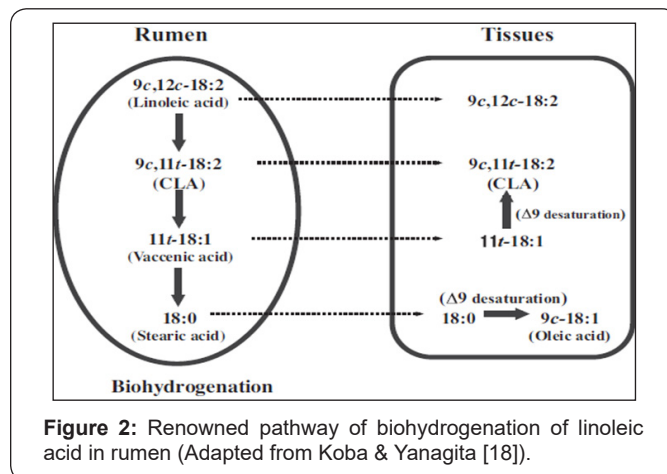


Figure 2: Renowned pathway of biohydrogenation of linoleic acid in rumen (Adapted from Koba & Yanagita [18]).

Lipid metabolism of luminal micro-organisms directly delivers CLA or important intermediate precursors of CLA on the way to the end product static acid. For instance, the renowned pathway of linoleic acid generates c9, t11-18:2 by isomeriation and t11-18:1 (trans-vaccenic acid) by further hydrogenation as intermediates. In general, a micro-organism does not cover the entire metabolism from the initial PUFA to the end product static acid but only part of it (Figure 2). Bacterial strain like, *Butyrivibrio fibrisolvens* release the enzymes linoleate isomerase and CLA reductase which isomerizes is double bonds of PUFA to form conjugated c/t double bonds and to hydrogenate these conjugated fatty acids. This process generates trans-vaccenic acid (t11-18:1) which is further hydrogenated to produce end product static acid (18:0) by other luminal bacteria [31]. PUFA like α-linolenic acid, the main sequence leads to conversion of trans-vaccenic to static acid with intermediates other than CLA [28,31,34].

Luminal pH acts an important parameter in isomeriation and biohydrogenation reaction [35]. In rumen, low pH alters the bacterial population [36] which affects the pattern of fermentation end products [37]. Griinari & Bauman [28] reported that the decreased rumen pH produce t10-18:1 instead of trans-vaccenic acid which generate t10, c12-18:2 as an intermediate product. Whereas, the pathways would not be same for all CLA isomers and other trans octadecenoic acids found.

CLA isomers were synthesized endogenously by Δ9-desaturase, which denaturants transvaccenic acid to c9, t11-18:2 [37-39]. Endogenous synthesis is the major source of c9, t11-18:2 in the milk fat which represents 78% and 64% of the total [28,38]. Desideration of vaccenic acid is the main source

of CLA in the muscle lipids based on the high correlations between CLA and trans-vaccenic acid [40]. Other CLA isomers commonly derive from other trans-18:1 isomers by the action of $\Delta 9$ -desaturase [28].

Whereas, endogenous synthesis was noticed in both the ruminants and non-ruminants [41-45]. The accessibility of trans-vaccenic acid is more in ruminants due to luminal biohydrogenation [35]. As well as endogenous synthesis trans-vaccenic acid was documented in humans but the predominant source of CLA comes from the dietary CLA intake with meat and meat products as well as milk and dairy products [45- 47].

Microbial synthesis of CLA: Biosynthesis methods have been employed to prepare a number of conjugated dienes. Researchers started synthesis of CLA isomers using bacterial enzymes from the date of discovery of bacteria which is present in the stomachs of ruminant would convert dietary unsaturated fatty acids present in the plant food sources into conjugated isomers. For example, the enzyme linoleate isomerase, isolated from the rumen anaerobic bacterium *Butyrivibrio fibrisolvens*, which isomerizes linoleic acid to mainly cis-9, trans-11 octadecenoic acid (also termed as rumenic acid) (US patent 6479683, 2002).

Lactobacillus delbrueckii ssp bulgaricus and *L. acidophilus* were tested for CLA production which was immobilized with chitosan and polyacrylamide with different pH. Chitosan (pH:8) and polyacrylamide (pH:7) immobilized with *Lactobacillus delbrueckii ssp* and *L. bulgaricus* has shown more CLA. Increase in cell count indicates the higher CLA production [48]. Oat lipids were used for microbial isomerization of CLA using *Propionibacterium freudenreichii ssp shermanii*. The hydrolyzed oat lipids were prepared in aqueous slurries by endogenous oat lipase. The slurry containing free LA was used as substrate for isomerization reaction. Nearly 80% of total CLA (c-9, t-11) formed [49].

CLA was synthesized from different forms of ricinoleic acid and castor oil with and without lipase (Lipase. M "Amano"10) using washed cells of *Lactobacillus plantarum* AKU 1009a as catalyst. After centrifugation, these washed cells were cultivated in MRS media containing 0.6g/l α -linolenic acid. Substrates with different concentration of 4.0mg/ml in form of free ricinoleic acid, ricinoleic acid in methyl ester form ricinoleic acid in methyl ester form with lipase, castor oil and castor oil with lipase. Substrate with free form of ricinoleic acid and castor oil with lipase produced maximum of 1.65mg/ml and 1.4mg/ml of CLA compared to other forms of substrates [50].

Concentrated LA and CLA (75%, c9, t11) solutions (0.1g/ml of water with 20% BSA) added to cultures of *Butyrivibrio fibrisolvens* A38 which was grown at 39 °C in basal medium under oxygen free CO₂. These cultures were harvested by centrifugation and cell pellets were prepared anaerobically or aerobically. Cell suspensions were incubated aerobically and an

aerobically, aerobic suspensions showed higher levels of CLA compared to anaerobic suspension [51].

Bacterial strain *Lactobacillus acidophilus* 1.1854 were tested for CLA production using alfalfa seed oil containing linoleic acid about 40% as substrate. Under optimal condition, 50% of conversion of LA to CLA was observed, which indicates the presence of linoleic acid isomerase activity in the culture [52]. Six strains of *Lactobacillus* species (*Lactobacillus acidophilus* L11, L12, L14, L15, *Lactobacillus fermentum* and *Lactobacillus router*) grown in MRS broth without linoleic acid at 37 °C with varying incubation time (3-42hrs). Among different strains of *Lactobacillus*, L11 produced highest enzymatic activity than other strains [53]. *Lactobacillus router* 55739 in MRS broth with and without linoleic acid (0.2%) were harvested and washed. The washed cells were added to buffer containing linoleic acid and without linoleic acid. The cells which have been grown without LA transformed more LA into CLA (c-9, t11) than cells grown with LA [54].

Chemical synthesis of CLA: The aim of chemical synthesis is to produce a fully characterized CLA composition with maximal biological activity. Some commercial preparations of CLA hold additional isomers with conjugated double bonds at the 8, 10 or 11, 13 positions [55]. Laboratory method of preparation of CLA consisting mainly of cis-9, trans-11 and trans-10, cis-12 isomers. (E.g. CLA prepared for experimental purposes contains c-9, t-11 (40.8-41.1%), t-10, c-12 (43.5-44.9%) and t-9, t-11/t-10, t-12 (4.6-10%) isomers [22,56,57].

Alkali isomerization method is widely used for producing CLA isomers chemically. In this method, homogeneous and heterogeneous catalysts were used. The main drawback of alkali isomerization of linoleic acid is the use of huge amount of strong basic potassium hydroxide or sodium methoxide [58]. Homogeneous catalysts are trialkyl phosphines (triphenylphosphine) chlororhodium and arene chromium carbonyl complexes [59], which enable lower reaction temperatures than 180-200 °C necessary for non-catalyzed systems. Homogeneous catalysts are soluble homogeneous metal complexes which are not eco-friendly and difficult to separate but heterogeneous catalysts are easy to separate and reuse. Hence, heterogeneous catalysts are advised for isomerization reaction of linoleic acid. Solvents containing hydrogen transfer agents are used for isomerization of methyl linoleate and rhodium and ruthenium supported with carbon was used as catalysts between 200 and 270 °C in different solvents [60,61].

In isomerization, the activities and selectivity's are reported to be dependent on the solvent used. For better selectivity, always non-polar solvents were used and high solvent/reactant ratio is preferred to avoid polymerization reaction. In case of hydrogenation, highly protective solvents such as methanol and isopropyl alcohol were used, which exhibits high activity and selectivity [62]. In CLA synthesis, isomerization and hydrogenation are two competing identical reactions. Isomerization occurs in

the absence of hydrogen preadsorption with the solvent being a hydrogen transfer agent, but the conjugation reaction is enhanced by catalyst reactivation under hydrogen. The catalyst holds chemisorbed hydrogen on the surface does not only increase the total conversion, but also increases the hydrogenation reaction of linoleic acid and CLA [63].

Metals like Ni, Pt, Pd, Rh, Ru and Ir and other transition elements like Cr, Mo, W, Cu and Zn were frequently used catalysts for hydrogenation [64]. The effect of $\text{RuCl}_2(\text{PPh}_3)_3$ on isomerization of industrial oils like rape seed oil, soybean oil and linoleic acid was investigated. The highest yields (88-91%) of conjugated isomers in the oils were obtained in the reactions carried out for 0.75-1.5hrs within the temperature range of 212-226 °C and with concentration of 0.03 to 0.110 wt% [65].

Larock et al. [66] employed 0.1mol% $[\text{RhCl}(\text{C}_8\text{H}_{14})_2]_2$, 0.25mol% $\text{PtCl}_2(\text{PPh}_3)_2$ or 0.5mol% $\text{RuHCl}(\text{CO})(\text{PPh}_3)_3$ to isomerizes the soybean oil to conjugated soybean oil under mild reaction condition. The $[\text{RhCl}(\text{C}_8\text{H}_{14})_2]_2$ catalyst provided high yield of conjugated linoleic acid without any hydrogenated product. Heterogeneous catalyzed conjugation reaction of linoleic acid over metals Ru, Ni, Pd, Pt, Rh, Ir, Os and Pt-Rh supported by carbon, Al_2O_3 , $\text{SiO}_2\text{Al}_2\text{O}_3$, H-MCM-41, MCM-22, H-Y and H- β (metal modified acidic zeolite) at mild reaction condition in batch wise manner with low polar solvents. Zeolite with increased selectivity favors the desired products trans-10, cis-12 CLA and the cancer inhibitor cis-9, trans-11 was obtained by the use of ruthenium on Al_2O_3 or ruthenium on carbon catalysts [63,67].

Andreas et al. [64] isomerizes technical grade of 55% linoleic acid to c9, t11-CLA and t10, c-12-CLA isomers in batch wise order at 165 °C over two series of supported metal catalysts (hydrogen reactivated and non-reactivated). Activated carbon and aluminum oxide, supported Ru, Pd, Os, Ir and Pt-Rh catalysts with 5% metal loading was screened. In metal catalyst Ru/ Al_2O_3 , chemisorbed hydrogen increased the isomerization rate for both the diluted and non-diluted systems. Overall ruthenium catalyst has shown higher selectivity than other metal catalysts.

Gangidi & Proctor [68] initiated the synthesis of CLA isomers using photoisomerization method. In this method linoleic acid methyl ester (5-10%) were dissolved in petroleum ether, benzene or carbon disulfide and then exposed to strong light source in presence of iodine as a sensitizer which gives a yield of 80% of CLA isomers. Jain & Proctor [69] designed a customized photochemical reaction system for CLA synthesis. The 144 hours of irradiation was initiated by transferring soybean oil to the reaction vessel using iodine as catalyst. A total CLA yield of 24% (w/w) total oil was obtained with 15% (w/w) iodine and trans-trans isomers (17.5%) and c9, t11 and t10, c12 CLA isomers (3.5%) is formed. Later they synthesized higher amount of CLA (75%) from soya oil which mainly composed of t8, t10-CLA, t9, t11-CLA and t10, t12-CLA [70].

Linoleic acid rich vegetable oils like flax seed oil, soybean oil, sunflower oil and safflower oil were used for the production of conjugated linoleic acid by photo catalytic isomerization system. Nearly, 700g of deaerated oil sample from all oil samples was transferred to the reaction vessel using 0.15% iodine as catalyst. A 168 hours of irradiation was conducted, 10ml of samples were collected every 24 hours of the irradiation for fatty acid analysis. Oils with highest linoleic acid produced more CLA in the order safflower oil > soya oil > corn > flax, sunflower oil does not produced any CLA even though it containing initial levels of LA [71].

Synthesis of conjugated linoleic acids over Ru supported on different zeolite varying in topology (ZSM-5, BETA, Y) Si/Al ratio and counter cation (H^+ , Na^+ , Cs^+). Combination of Ru/ Cs -USY with Si/Al ratio of 40 identified as most active and selective catalyst for effective isomerization of methyl linoleate to CLA at 165 °C, produced 0.7g of CLA/liter of solvent/ minute [72]. Silva Ramirez et al. [73] synthesized c9, t11 and t10, c12 CLA isomers by microwave irradiation. The experiment was conducted with different set of variables like solvent/LA mass ratio (1:1-6:1), catalyst/LA mass ratio (0.25:1-0.6:1), temperature (160-180 °C), catalyst type (KOH or NaOH) and reaction time. The best optimum conditions for synthesizing CLA isomers (91.21% equimolar ratio) was NaOH at a catalyst/LA mass ratio of 0.5:1 and solvent/LA mass ratio of 1:1 at 160 °C during 4min.

Conclusion

Conjugated linoleic acid is a mixture of positional and geometric isomers of linoleic acid with two conjugated double bonds at various carbon positions in the fatty acid chain. C-9, t-11 is the most prevalent isomer comprising 80-90% of the total CLA in food products from ruminants. CLA can be synthesized in tissues endogenously by Δ^9 -desaturase enzyme and as intermediate by partial biohydrogenation of unsaturated fatty acids present in the dietary source of ruminants [74-76]. Many microbial strains mostly *Lactobacillus spp* and *Butyrivibrio spp* were employed for CLA synthesis. Increasing market demand and GRAS status for CLA in 2008 by FDA, researchers and scientists has focused their interest on bulk production of CLA using many metal catalysts and by photo-isomeric methods. It is highly appreciated that, research on CLA synthesis should focus in discovering more number of other bacterial strains and many environmental friendly heterogeneous catalysts with increased yield of specific CLA isomers.

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