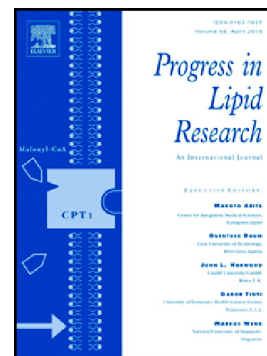# Accepted Manuscript

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Bacterial conjugated linoleic acid production and their applications

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1 **ABSTRACTS**

2 Conjugated linoleic acid (CLA) has been shown to exert various potential physiological

3 properties including anti-carcinogenic, anti-obesity, anti-cardiovascular and anti-diabetic

4 activities, and consequently has been considered as a promising food supplement. Bacterial

5 biosynthesis of CLA is an attractive approach for commercial production due to its high

6 isomer-selectivity and convenient purification process. Many bacterial species have been

7 reported to convert free linoleic acid (LA) to CLA, hitherto only the precise CLA-producing

8 mechanisms in *Propionibacterium acnes* and *Lactobacillus plantarum* have been illustrated

9 completely, prompting the development of recombinant technology used in CLA production.

10 The purpose of the article is to review the bacterial CLA producers as well as the recent

11 progress on describing the mechanism of microbial CLA-production. Furthermore, the

12 advances and potential in the heterologous expression of CLA genetic determinants will be

13 presented.

14 **Key words**: conjugated linoleic acid, bacterial CLA-producers, CLA bioconversion

15 mechanism, microbial cell factories

1. Introduction

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid (C18:2, *c*9,*c*12) with conjugated double bonds. CLA has attracted great interest in recent decades due to its biological and physiological benefits [1, 2], including anti-carcinogenesis [3-5], anti-obesity [6-9], anti-inflammation [10, 11], anti-diabetic [12-14] as well as bone formation-promoting properties [15, 16]. Many bacteria have been reported to convert free LA into CLA, such as *Butyrivibrio fibrisolvens* [17], *Lactobacillus reuteri* [18], *L. plantarum* [19, 20], *L. casei* [21], *L. acidophilus* [22], *Bifidobacterium breve* [23], *B. longum* [24], *Propionibacterium acnes* [25]*, P. freudenreichii* [26] and *Clostridium sporogenes* [27].

Most commercial CLA is produced via chemical isomerization of LA, however, the chemical process will result in unexpected by-products, which are mostly harmful [28]. Although CLA has different isomers, many studies have confirmed that only *c*9,*t*11-CLA, *t*9,*t*11-CLA and *t*10,*c*12-CLA are considered as the isomers with beneficial activities. Considering the usage of CLA for medical purposes, a safe isomer-selective process is required. Microbial CLA seems to be a perfect approach, although the CLA concentration typically produced during microbial fermentation is insufficient for commercial purposes. A potentially better alternative is the use of genetically modified organism (GMO) recombineered to overproduce CLA with high efficiency. The advances in the identification of CLA-producing mechanisms among some strains have prompted the development of recombinant technology in CLA production such as *Escherichia coli* [29] and *Yarrowia lipolytica* [30, 31].

This article will review the current knowledge on microbial CLA producers and the precise mechanisms for bacterial CLA production, as well as recombinant DNA technology for industrial CLA production.

2. Bacterial CLA producers

According to the major CLA isomers, the strains that can produce CLA can be divided into two groups: 9,11-CLA (*c*9,*t*11-CLA and *t*9,*t*11-CLA) and *t*10,*c*12-CLA. Some typical strains with high conversion rates are listed in Table 1.

2.1 *c*9,*t*11-CLA and *t*9,*t*11-CLA producers

2.1.1 Rumen bacteria

Rumen bacteria can bio-hydrogenate some unsaturated fatty acids to *trans*-vaccenic acid

46   or stearic acid with CLA as intermediate [32]. Since demonstration of the capacity of *B.*

47   *fibrisolvens* A38 to produce CLA efficiently among rumen bacteria, it has been widely used as

48   a model for CLA production in rumen bacteria [33-35]. It is reported that 40% of LA is

49   converted to CLA by this strain, of which 95% is the *c*9,*t*11-CLA. Furthermore, accumulation of

50   CLA is inhibited by such factors as high concentration of LA [36], aerobic conditions [36] as

51   well as glycolytic inhibitors [37]. CLA reductase activity has been identified as another

52   important factor influencing CLA production. For example, *B. fibrisolvens* TH1 exhibits high

53   CLA isomerase activity, but the accumulation of CLA is low, mostly due to high reductase

54   activity, catalyzing the conversion of CLA into *trans*-vaccenic acid [38]. In contrast, the

55   accumulation of CLA occurs in *B. fibrisolvens* MDT-5 due to the absence of CLA reductase

56   activity. Thus, the latter strain could be considered to be an ideal probiotic for animal nutrition

57   for its high CLA production capability [39].

58   2.1.2 *Lactobacillus*

59   Lactobacilli have attracted more attention than other CLA-producing strains due to their

60   health-promoting effects. Many species of lactic acid bacteria have been reported to possess

61   the ability to produce CLA. *L. reuteri* was the first species of lactic acid bacteria reported with

62   high CLA production capability [40]. Subsequently, Lee et al. [41] found that immobilized *L.*

63   *reuteri* cells could accumulate 5.5 times more CLA than that obtained from the conversion by

64   free washed cells. Furthermore, glycocholate which occurs in humans is shown not to

65   influence CLA production by *L. reuteri* ATCC 55739 [42]. The bioconversion capability of CLA

66   in *L. reuteri* at different conditions was investigated [43], and highest concentration of CLA was

67   obtained in broth containing 20 mg/l free LA aerobically at 10 ^{o}C for 30 h. *L. plantarum* is the

68   most widely reported species among lactobacilli with high CLA-production ability. In 2002,

69   Kishino et al. identified some strains that have the ability to generate CLA, in which *L.*

70   *plantarum* AKU1009a showed the highest conversion rate, with up to 85% of LA being

71   converted into *c*9,*t*11-CLA [19]. CLA could also be accumulated at a level of up to 2700 mg/l by

72   *L. plantarum* JCM1551 with ricinoleic acid (12-hydroxy-*cis*-9-octadecenoic acid) as the

73   substrate [44]. It is also reported that both the growing culture and washed cells of *L.*

74   *plantarum* ZS2058 could effectively convert LA into CLA, at rates of 54.3% for growing cultures

75   and 46.75% for washed cells, respectively [45]. Additionally, washed cells and substrate

76 concentration, manipulation of the content of yeast extract and glucose in MRS broth

77 increased the CLA productivity significantly in *L. plantarum* [46]. Linoleic acid could be

78 converted into different hydroxyl fatty acids such as 10-HOE, 10,13-diHOA, and

79 13-hydroxy-*cis*-9-ocatadecenoic acid (13-HOE). For instance, *L. reuteri* LTH 2584 [47], *L.*

80 *sanfranciscensis* ATCC27651[48, 49], *L. hammesii* DSM16381 [47-49], *L. spicheri* LS38 [47],

81 *L. rhamnosus* LGG [50], *L. plantarum* ST-III [50], *L. acidophilus* NCFM [50], *L. plantarum*

82 AKU1009a [19, 51-55], *L. plantarum* ATCC 8014 [56], *L. plantarum* ZS2058 [45] and *L.*

83 *acidophilus* AKU1137 [28, 57] could generate 10-HOE. In the CLA producers, 10-HOE was

84 accumulated during CLA production in *L. acidophilus* AKU1137, *L. plantaurm* AKU1009a and,

85 *L. plantarum* ZS2058 and *L. plantarum* ATCC8014, and was confirmed as CLA-generation

86 intermediates. In hence, the enzyme catalyzing 10-HOE production should be essential for

87 CLA production in those strains. *L. acidophilus* NBRC 13951 [58] could convert LA to 10-HOE

88 and 13-HOE, while *L. plantarum* TMW 1.460 [47, 48], *L. acidophilus* LMG 11470 [59, 60], and

89 *L. acidophilus* AKU1137 [61] could metabolize LA into three hydroxyl fatty acid including

90 10-HOE, 13-HOE,10,13-diHOA. Heretofore, no evidence shows 13-HOE or 10,13-diHOA are

91 involved in CLA production.

92 Washed cells of *L. acidophilus* La-5 accumulated CLA in the cells, but not in the culture

93 medium. In addition, absence of oxygen could only influence the ratio of different CLA isomers

94 produced, but not total CLA concentration. Tween 80 has been shown to be effective to

95 promote growth and c9,t11-CLA production efficiency of *L. acidophilus* F0221 in the presence

96 of bile salts [62]. Similar to *L. acidophilus*, immobilized cells of *L. delbrueckii* subsp. *bulgaricus*

97 onto polyacrylamide could significantly increase the content of CLA compared with that by free

98 washed cells [63]. Other *Lactobacillus* strains have shown high abilities to produce CLA, such

99 as *L. casei*, *L. pentosus* and *L. brevis* [19]. The application of *L. casei* for the synthesis of CLA

100 in both hen eggs and broiler meat cuts were demonstrated, suggesting this strain as a suitable

101 probiotic for such application [64].

102 2.1.3 *Bifidobacterium*

103 The ability of *Bifidobacterium* to produce CLA was firstly reported by Coakley and

104 colleagues [65], in which nine of fifteen strains presented high CLA-producing activity with

105 c9,t11-CLA as the predominant isomer produced and members of *B. breve* species as the

106 most efficient for CLA production. One hundred and fifty strains of bifidobacteria strains were

107 isolated from human intestines [66], and four isolates showed 80% conversion of LA to CLA in

108 MRS broth. Among these strains, *B. breve* LMC 017 could convert up to 90% of linoleic acid or

109 78.8% of monolinolein into CLA. *B. breve* LMC520 is reported to produce CLA with maximal

110 bioconversion rate up to 90% [23]. Thirty six bifidobacteria strains were assessed for CLA

111 production [67] and consequently four *B. breve strains* were discovered to transform LA into

112 CLA ranging from 19.5% to 53.5%. More than 70% of CLA isomers produced by *B. breve* were

113 *c*9,*t*11-CLA, meanwhile approximately 38% of CLA isomers were *t*9,*t*11-CLA in *B. breve* LMG

114 13194. *B. longum* is another high CLA production species among the genus *Bifidobacerium*.

115 Rapid screening of CLA-producing bifidobacteria was established by Barrett et al. [24]. With

116 this method, four *B. longum* strains, isolated from feces, were found to convert more than 20%

117 of free LA to CLA [24]. *B. longum* DPC6320, demonstrated 43.89% *c*9,*t*11-CLA conversion,

118 while *B. longum* DPC6315 could convert only 11.02% of free LA into *c*9,*t*11-CLA [68]. Another

119 study showed that *B. longum* could increase the content of CLA in the cheese by 20.44% [69].

120 *B. animalis* Bb12, one of the most widely used probiotics, could transfer 27% of free LA into

121 *c*9,*t*11-CLA in MRS broth [65]. *B. animalis* BLC showed the best CLA production with free LA

122 as substrate while *B. animalis* Bb12-1 demonstrated the highest conversion rate of CLA with

123 ricinoleic acid as substrate [70]. Additionally, *B. dentium* NCFB 2243 could convert 29% of LA

124 into 9,11-CLA [47]. The CLA conversion rate of *B. bifidum* CRL 1399 was up to 24.8% in MRS

125 broth [71]. Gorrisen and colleagues [67] discovered that *B. bifidum* LMG 10645 could produce

126 CLA from LA with conversion rate of 40.7%. *B. animalis* subsp.*lactis* Bb-12 [50], *B. breve*

127 NCIMB 702258 [72] could produce 10-HOE during CLA generation.

128 2.1.4 *Propionibacterium*

129   The first CLA-production *Propionibacteria* was reported by Verhulst and colleagues [26],

130 in which *P. freudenreichii* subsp. *freudenreicbii*, *P. freudenreichii* subsp. *shermanii*, *P.*

131 *acidi-propionici* and *P. tecbnicum* could produce *c*9,*t*11-CLA. Jiang et al. [73] analyzed dairy

132 starter cultures for the capability to produce CLA from free LA in MRS broth. Three

133 *Propionibacteria* strains were discovered to generate CLA with high efficiency. *P. freudenreichii*

134 subsp. *freudenreichii* Propioni-6 Wiesby showed the highest CLA-production capability (35.3%

135 conversion). *P. shermanii* AKU1254 could produce 0.11 g/l CLA in reaction mixture with 4 g/l

136   free LA, and the CLA produced was the mixture of *c*9,*t*11-CLA and *t*9,*t*11-CLA [19].

137   2.1.5 *Clostridium*

138   Several strains of *Clostridium bifermentans*, *C. sporogenes* and *C. sordelli* were shown to

139   hydrogenate LA into *trans*-vaccenic acid *in vitro* with *c*9,*t*11-CLA as intermediate [27]. Peng et

140   al. [74] showed that *c*9,*t*11-CLA accumulated in *C. sporogenes* ATCC 22762 within 30 min and

141   then *t*9,*t*11-CLA and *t*10,*t*12-CLA increased at the expense of *c*9,*t*11-CLA until these reached

142   the same level.

143   2.1.6 Other *c*9,*t*11-CLA producers

144   Other strains showed the ability to produce CLA. *Lactococcus lactis* subsp. *cremoris*

145   CCRC12586, *L. lactis* subsp. *lactis* CCRC 10791 and S. thermophilus CCRC 12257 were

146   reported to convert free linoleic acid in skim milk plus 12% free linoleic acid [22]. Additionally,

147   some other *Lactococcus* [75], *Streptococcus* [76], *Leuconostoc* and *Pediococcus* [76, 77]

148   strains have shown the capability to produce CLA with different substrates.

149   **2.2   *t*10,*c*12-CLA producers**

150   Bacterial species which could produce *t*10,*c*12-CLA are less common than those

151   producing *c*9,*t*11-CLA. *T*10,*c*12-CLA is the isomer presenting significant benefits on

152   anti-obesity [78]. *P. acnes* has been shown to convert LA into *t*10,*c*12-CLA. Verhulst and

153   colleagues firstly reported the ability of *P. acnes* to produce *t*10,*c*12-CLA, including strain

154   ATCC 6919, ATCC 6921, VP1 162, VPI 163, VPI 164, VPI 174, VPI 186, and VPI 199 [26]. *P.*

155   *acnes* ATCC 6919, could also catalyze the production of *t*10,*c*12-CLA from linoleic acid at high

156   level [79].

157   Additionally, *t*10,*c*12-CLA could be also produced from linoleic acid by *Megasphaera*

158   *elsdenii* [36]. *M. elsdenii* YJ-4 could catalyze 35% of free LA into CLA, among which the

159   percentage of *t*10,*c*12-CLA is up to 85% [80] . Peng et al.[74] showed that *C. sporogenes*

160   ATCC 22762 could generate *t*10,*t*12-CLA at a low level while *c*9,*t*11-CLA was the major isomer

161   produced.

162   While *c*9,*t*11-CLA was the major isomer in lactic acid bacteria, *t*10,*c*12-CLA isomer could

163   be produced by some lactic acid bacteria strains [21]. Enzymes were purified from

164   *Lactobacillus* strains to determine their capability for CLA production and consequently, *L.*

165   *rhamnosus* PL60 and *L. pentosus* IFO 12011 were identified to produce considerable

166   *t*10,*c*12-CLA isomer [81]. CLA produced by *L. plantarum* NCUL005 consists of 32.2%

167   *c*9,*t*11-CLA isomer and 67.8% *t*10,*c*12-CLA isomers [82]. In *L. reuteri* ATCC55739,

168   *t*10,*c*12-CLA could make up 41% of the final CLA isomers [18]. As well as *P. freudenreichii* with

169   high CLA production ability could accumulate a smaller amount of *t*10,*c*12-CLA when they

170   produce amount of *c*9,*t*11-CLA and *t*9,*t*11-CLA from linoleic acid [73].

3. Role of CLA production in bacteria

172   The role of conjugated fatty acids in bacterial cells is unclear, but a number of proposals

173   have been made. For example, it has been proposed that bio-hydrogenation is a means for

174   anaerobic bacteria to dispose of reducing power [83]. Bacterial cells capable of isomerizing

175   linoleic acid to CLA exhibit greater tolerance to linoleic acid, compared with bacteria that

176   exhibit non-CLA production. In a research by Jiang et al [73], it was reported that the majority

177   of CLA-producing *Propionibacteria* were those inhibited by the presence of high

178   concentrations of free linoleic acid, and a positive correlation between microbial CLA

179   production and tolerance to linoleic acid was observed among the CLA producing strains. This

180   suggested that microbial CLA production may be a detoxification mechanism for the bacterial

181   cell. Linoleic acid has been shown to be toxic to many bacteria, as shown by lack of ability to

182   grow in the presence of the fatty acid [21, 73, 84, 85]. When cultured in the presence of the

183   more rigid CLA molecule, as compared with linoleic acid, bacterial cells exhibited superior

184   growth, while growth was unaffected by stearic acid [85]. In general, long chain fatty acids with

185   a higher degree of unsaturation are reportedly more inhibitory bacterial cell growth than fatty

186   acids of the same chain length but with fewer unsaturated double bonds [86]. The presence of

187   linoleic acid and CLA in the medium has been shown to up-regulate the molecular chaperone

188   *GroEL* in *B. fibrisolvens* by 6.9 and 5.5-fold, respectively [87]. This induced expression of

189   *GroEL* is believed to be a non-specific response to stress rather than a specific mechanism

190   enabling *B. fibrisolvens* to withstand the toxic effects of linoleic acid and CLA.

4. Bacterial CLA-production mechanism

192   A number of species and strains generating CLA were reported, however, heretofore

193   mechanisms for CLA bioconversion have not been elucidated for each species.

4.1 Mechanism for *c*9,*t*11-CLA production

4.1.1 Rumen bacteria

8

196   Two processes involved in the bioconversion of LA into stearic acid <mark>exist</mark> in the mixed

197   rumen bacteria. The first process is from LA to monoenoic acid, followed by the process from

198   monoenoic acid to stearic acid [88, 89]. The enzyme which catalyzes the conversion of linoleic

199   acid to c9,t11-CLA is linoleate isomerase. The isomerase from *B. fibrisolvens* is reported to be

200   membrane-bound and have maximum activity within a narrow substrate concentration range

201   (α-linolenic aicd and γ-linolenic acid), and to have absolute specificity for a substrate

202   containing a free carboxyl group and a c9,c12 diene bonding system. Cofactors seem to be not

203   essential for the isomerase because enzymatic activity would not be influenced by passing

204   through corresponding chromatographic columns or the addition of CoA, ATP, Mg^{2+}, ADP, AMP

205   and NAD^{+} [35]. Isomeration of LA into CLA by the crude enzyme extracts was not affected by

206   the aerobic condition, even though anaerobic condition was essential for intact cells [89, 90].

207   The preferred ω (omega) chain length was shown to be 6 carbons, as heptadecadienoic acid

208   (C17:2, ω7) was isomerized at only half the rate of LA [35, 91]. Kepler et al. [91] proposed a

209   model for the isomerization of LA by linoleate isomerase, in which the substrate binds initially

210   to the enzyme in a hydrophobic pocket. The binding involves interaction of the π-electrons of

211   the substrate double bond with an electrophilic group on the enzyme and hydrogen bonding of

212   the undissociated carboxyl group of the substrate with an electronegative center in the enzyme

213   (Fig. 1) [92]. It was concluded that the hydrogen added to carbon 13 was derived from water,

214   which suggests that the hydration added to carbon 13 was derived from water, which suggests

215   that the hydration-dehydration mechanism generates ricinoleic acid as an intermediate,

216   although no hydroxyl fatty acids were detected.

217   Hydrogenation of CLA by reductase activity could influence the amount of the CLA in

218   these strains. CLA reductase is approximately 60 kDa, which could convert the CLA to the t9 or

219   t11 ocatdecenoic acid [93, 94]. Fukuda et al. demonstrated that addition of saturated fatty acid

220   could stimulate the activity of isomerase, while the activity of CLA reductase would increase

221   with the addition of unsaturated fatty acid [95]. Fukuda et al. also discovers that *cla-r* is

222   responsible for the isomerase activity [39, 93]. Though many properties of the isomerase have

223   been clarified, the purification of linoleate isomerase from the *B. fibriosolvens* is still

224   unsuccessful. More effort should be taken to obtain the crystal structure of isomerase in order

225   to clarify the essence of the isomerization.

226 4.1.2 *Lactobacillus*

227   Putative linoleate isomerase in lactobacilli is considered as the key factor involved in the

228 bioconversion of LA into CLA, similar to that in the rumen bacteria and *Propionibacterium*, in

229 which no intermediates were accumulated. Rosson et al. isolated and characterized a special

230 enzyme from *L. reuteri* ATCC55739, which was originally identified as isomerase [96], and the

231 size of which was reported as 67 kD with an optimum pH of 6.8-7.5 [97]. Moreover, this protein

232 is identified to be homogenous to myosin-cross-reactive antigen (MCRA), which is universal

233 among bacteria. However, when the gene was cloned into various expression systems, no

234 CLA was produced, although recombinant *Bacillus subtilis* produced a fatty acid with the same

235 molecular weight as a hydroxylated linoleic acid derivative at the expense of linoleic acid [97].

236 High homology between the purified linoleate isomerase from *L. reuteri* and the relationship

237 between hydroxyl fatty acid and CLA have already been discovered. Volkov et al. firstly

238 demonstrated that MCRA from *S. pyogenes* is a fatty acid hydratase not a linoleate isomerase

239 [98]. In addition, the crystal structure of MCRA from *L. acidophilus* NCFM was analysed [99].

240 FAD was found to be loosely linked to MCRA. Furthermore, four intricately connected domains

241 were observed in the crystal structure, and three domains (domain1, domain2 and domain 3)

242 are responsible for the formation of a hydrophobic substrate channel, similar to that found in

243 several flavin-dependent amino-oxidases (Fig. 2). With linoleic acid as substrate,

244 10-hydroxy-*cis*-12-octadecenoic acid (10-HOE) and 10,13-dihydroxy-octadecanoic acid

245 (10,13-diHOA) were both produced by the purified MCRA from *L. acidophilus* NCFM. MCRA is

246 successfully purified from *L. plantarum* AKU1009a [51-55, 100], identified as FAD-independent

247 hydratase with NADH as the activator, similar to other MCRAs [50, 98]. It is also reported in

248 this study that $FADH_2$, produced by hydrating FAD by NADH, may be the actual activator for

249 the reaction. The hydratase seems to preferentially act with the substrate coupled with

250 ethylenic bond in the hydration reaction, such as the linoleic acid, oleic acid and that with

251 hydroxyl at $\Delta 10$ position in the dehydration reaction. The result is similar to that of MCRA

252 isolated from *Elizabethkingia meningoseptica* [101], *S. pyogenes* [98], and *B. breve* [72].

253 Further studies performed in our lab demonstrate that MCRA in different lactic acid bacteria

254 functioned as hydratase not a linoleate isomerase [50]. Similar results were obtained in other

255 researches [102, 103]. Therefore, the mechanism for CLA production in lactobacilli remained

256   unclear.

257   Multiple-step reactions for CLA production was hypothesized and several intermediates

258   were determined respectively. Ogawa et al. [28] reported that following incubation with washed

259   cells of various cultures; two different isomers of CLA were detected (*c*9,*t*11-CLA and

260   *t*9,*t*11-CLA) together with two hydroxyl fatty acids, 10-hydroxy-*trans*-12-octadecenoic acid and

261   10-HOE. To elucidate the mechanism of CLA production, *L. acidophilus* AKU 1137 was found

262   to accumulate hydroxyl fatty acids before CLA production, which decreased rapidly as the

263   reaction proceeded (Fig. 3). This finding suggests that the two hydroxyl fatty acids are

264   intermediates in CLA production, starting with hydration of linoleic acid to

265   10-hydroxy-octadecenoic acid and subsequent dehydrating isomerization of the hydroxyl fatty

266   acid to CLA. Furthermore, hydroxyl fatty acids when used as substrate were converted to CLA

267   by *L. acidophilus*. Free ricinoleic acid was a substrate for CLA production for a wide range of

268   LAB [104]. In 2011, Kishino et al. successfully isolated three proteins from *L. plantarum*

269   AKU1009a, in which one was membrane-protein involved in the forming

270   10-hydroxyl-*cis*-12-octadecenic acid from LA and the other two proteins existed in the

271   cytoplasm. Together with these three proteins, LA could be converted to CLA [52], and those

272   proteins are highly homogenous to MCRA, short-chain dehydrogenase/oxidoreductase and

273   acetoacetate decarboxylase of *L. plantarum* WCFS1, respectively. Based on the N-terminal

274   sequencing, three genes involved in the production of CLA from linoleic acid, *cla-hy, cla-dh*

275   *and cla-dc*, were identified [51]. For further analysis, the three genes were heterologously

276   expressed in *E. coli* and with the mixture of the recombinants CLA was generated successfully,

277   which was the first report for the confirmed mechanism of CLA production in lactic acid

278   bacteria. Later research in the group, the whole metabolism of LA was illustrated; with hydroxyl

279   fatty acids and oxo fatty acids as intermediates, LA could be converted into CLA and oleic acid,

280   those metabolites were catalyzed by CLA-HY (10-LAH), CLA-DH, CLA-DC, and CLA-ER [53].

281   This pathway includes the process of hydration, dehydration and double-bond immigration,

282   and the precise pathway was described as follows (Fig. 3). According to position of hydroxyl

283   group, the enzymes catalyzing linoleic acid hydration could be divided into two groups:

284   linoleate 10-hydratase (10-LAH) and linoleate 13-hydratase (13-LAH), in which 10-LAH is

285   defined as oleate hydratase (EC 4.2.1.53). Interestedly, the enzymes converting LA to hydroxyl

11

fatty acids are belonged to MCRA family, which means MCRA is multifunctional. MCRA from *L. planarum* AKU1009a [51-55], *L. plantarum* ZS2058 [45], *L. plantarum* ATCC8014 [56], *L. reuteri* LTH2584 [47], *L. hammessi* DSM16381 [47], *L. rhamnosus* LGG [50] and *L. plantarum* ST-III [50] were cloned and identified as FAD-dependent linoleate 10-hydrase, in which FAD was confirmed as an essential factor. Several linoleate 13-hydtastases were characterized as well. MCRA from *L. acidophilus* NCFM was characterized [50], and its crystal structure was analyzed, with the purified protein 10-HOE and 10,13-diHOA could be produced from LA [99]. In addition, MCRA of *L. acidophilus* LMG 11470 was characterized with multiple functions as well which could convert LA to 10-HOE and 13-HOE [59]. Current knowledge on the linoleate 13-hydratase shows that they are not FAD-dependent. However, how 10-LAH and 13-LAH generated 10,13-diHOA remains unclear.

Similar results are observed in our lab in *L. plantarum* ZS2058, a strain with efficient production of CLA. 10-hydroxyl-*cis*-12-octadeneic acid, 10-oxo-*cis*-12-octadeneic acid, 10-oxo-*trans*-octadeneic acid are detected, which is considered as the intermediates during the isomerization of LA into CLA. Moreover, the corresponding genes were also identified in detail [45].

A more recent publication reported that 10-HOE could be transferred into *c*9,*t*11-CLA directly by enolase (Fig. 3) [105]. This novel enzyme could not recognize LA as the substrate instead 10-HOE. These results enriches our understanding of producing CLA among *L. plantarum*, which indicated multiple or alternative mechanisms for CLA production in lactobacilli.

4.1.3 *Bifidobacterium*

The information about mechanism of CLA production in *Bifidobacterium* is limited. Rosberg-Cody et al. [72] firstly reported a putative linoleate isomerase gene in *B. breve*, which is highly homogenous to *mcra* in *L. reuteri* ATCC 55739. This corresponding enzyme is identified as FAD-dependent protein, catalyzing reaction from the LA to 10-hydroxy-*cis*-12-octadecacenic acid, similar to our finding in *B. lactis* Bb12 [50]. It's reasonable to speculate that 10-hydoxy-*cis*-12-octadeceneic acid might be the first intermediate in bifidobacterial CLA generation. However, after *mcra* was knocked out, MCRA in the *B. breve* has been reported to be only active for oleate hydrogenation nor CLA

316 production in the *mcra* knock-out mutant [106]. The mechanism for CLA production in

317 *Bifidobacterium* remains unclear, which needs further investigation.

318 4.1.4 *Clostridium*

319 Linoleic acid could be isomerized by *C. sporogenes* to *c*9,*t*11-CLA [27], followed by

320 accumulation of *t*9,*t*11-CLA and *t*10,*t*12-CLA isomers at the expense of *c*9,*t*11-CLA isomers

321 [74]. *C. sporogenes* linoleate isomerase has already been purified, identified to be

322 membrane-associated and shown to be unstable, especially being solubilized by detergents.

323 Similar to the substrate specificity of isomerase in *B. fibrisolvens*, *P. acnes* and *L. plantarum*,

324 *cis* double bond at the *c*9 and *c*12 position of C18 polyunsaturated fatty acids with free

325 carboxyl group seems to be necessary in *C. sporogenes.* Furthermore, no external cofactors

326 or energy sources were required for isomerization. However, the corresponding gene(s) has

327 not been identified yet and detailed characterization of this enzyme is still unclear.

328 **4.2 Mechanism for *t*10,*c*12-CLA production**

329 In contrast to linoleate isomerase responsible for production of *c*9,*t*11-CLA in lactic acid

330 bacteria, polyunsaturated fatty acid isomerase (PAI) involved in the bioconversion of LA to

331 *t*10,*c*12-CLA in *P. acnes* has been clearly elucidated [79, 107, 108]. Bioconversion of CLA by *P.*

332 *acnes* is confirmed to be one-step and nonredox isomerization, in which linoleic acid is directly

333 catalyzed into *t*10,*c*12-CLA [79] . PAI is also identified to be FAD-dependent, however, with no

334 requirement for reducing power (NADH or NADPH) and also to be soluble protein with 424

335 amino acid, different from those isolated from *B. fibrisolvens* [17], *L. plantarum* [51] and *C.*

336 *sporogenes* [74]. Furthermore, crystal structure of PAI has already been elucidated, in which

337 PAI and PAI-LA complexes are purified and then determined (Fig. 4) [108, 109]. Three

338 intricately connected domains are found in PAI, in which FAD could be nonvalently linked to

339 domain 1 and positively charged patches generated by several Lys and Arg residues could be

340 localized near the channel entrance in domain 3 serving as an initial recognition site for the

341 carboxylate group of the fatty acid. It has been noted that isomerization of LA by PAI is initiated

342 by abstracting a hydrogen anion at C11 directly toward the atom N15 of FAD and then

343 carbocation is produced, served as the intermediate stabilized by π-cation and helix dipole.

344 *T*10,*c*12-CLA is then generated by addition of the hydrogen at C9 and reproduction of FAD.

345 The PAI represents the first report of a crystal structure reported for a fatty acid isomerase,

which reveals a unique gating mechanism for substrate specificity, due to the conformational changes in the hydrophobic channel toward the active site. The length preference for C18 fatty acids can thus be explained by the fixed distance between the FAD and the substrate carboxylate (11 carbon atoms in the case of linoleic acid and linolenic acids) [108, 109].

5. Microbial cell factories for *t*10,*c*12-CLA production

The original bacterial CLA producers only recognize the free fatty acids as substrate whereas high concentration of free fatty acids could inhibit the growth of bacteria. Therefore those microbial CLA producers are not suitable for industrial purpose. With the genetic determinants for CLA production available, genetic engineering and metabolic engineering might be alternative to serve as microbial cell factories for commercial CLA production.

5.1 Bacteria

Escherichia coli is among the most commonly used host in genetic engineering. Rosberg-Cody et al. demonstrated that *E. coli* cells carrying the construct co*PAI* gene converted about 40% of LA [29]. Moreover, IPTG induction condition must be developed otherwise linoleate isomerase expressed in *E. coli* would be no activitity [79]. Further studies demonstrated that fed batch fermentation could be considered as an effective method to improve the expression of PAI with significant activity. Except this typical C9 isomerase, C12 linoleate isomerase was also successfully hetero-expressed in *E.coli* [110]. All these trials would provide an effective product on process of CLA for the medical and nutritional purposes. However, the final concentration of CLA was limited by the resistance of *E. coli* in fatty acids. PAI from *P. acnes* was cloned and successfully heterologeously expressed in *L. lactis* NZ9800 [29]. Approximately 50% of linoleic acid could be converted to *t*10,*c*12-CLA by the recombinants, which could also significantly inhibit the growth of SW480 cancer cells. Ingestion of recombinant *L. paracasei* NFBC 338 with PAI gene could lead to 4-fold increase in *t*10,*c*12 CLA in adipose of mice and 2.5-fold increase in liver [78]. The cost for industrial fermentation of *L. lactis* is expensive, which results in products without price advances.

5.2 Yeast

Baker's yeast. *Saccharomuces cerevisiae* is another well studied organism for metabolic engineering. For example, it has been metabolically engineered to produce artemisinic acid and amorpha-4,11-diene [110]. Expression of PAI genes in *S. cerevisiae* has also been

376 successful in recent years, in which the amount of CLA could be up to 57% of total free fatty

377 acid [107]. *S. cerevisiae* seems to be a potential microbial cell factory for CLA production.

378 **Oleaginous yeast.** An oleaginous yeast, *Yarrowia lipolytica*, is naturally capable of

379 accumulating lipids to levels exceeding half of dry cell weight (DCW), and with appropriate

380 genetic modifications, such as abolishing the lipid turnover pathway, lipid accumulation could

381 exceed 80 % of DCW [111]. *Y. lipolytica* could accumulate >90 % of neutral lipids in the

382 triacylglycerols (TAG). These unique features of *Y. lipolytica*, together with the availability of

383 genetic tools, have already attracted great interest in the usage for bio-oil production. With a

384 series of genetic modification (overexpression and knockout), the final engineered yeast lipid

385 comprises EPA at 56.6% by weight, which was the highest among known EPA sources [112].

386 Our research firstly reported the production of *t*10,*c*12-CLA by recombinant *Y. lipoltica* with PAI

387 overexpression. It is notable that with multi-copy integration the yield of CLA is appropriately

388 30 times in yeast carrying the codon-optimized gene than that carrying the native gene, which

389 is approximately 7.0 mg/l for the former strains. Lately the amount of *t*10,*c*12-CLA in *Y.*

390 *lipolytica* Polh was increased to the level of up to 10% by co-expressing delta 12-desaturase

391 gene together with the co-PAI multi-copy integration [30]. In addition, permeabilization of the *Y.*

392 *lipolytica* by freeze/thawing has also been identified to significantly increase the amount of

393 *t*10,*c*12-CLA up to 15.6 g/l and also to remain the extracellular production of

394 *t*10,*c*12-conjugated linoleic acid above 10 g/l, and now with LA adding the CLA yield is around

395 22 g/l (Fig. 5) [113]. A recent research by other researchers tried to produce CLA within *Y.*

396 *lipolytica* as well, and the final concentration of CLA is approximately 302 mg/l [31]. Hence, *Y.*

397 *lipolytica* could be considered as the suitable microbial cell factory for CLA production via

398 bioengineering. Additionally, several technologies, including various fermentation

399 configurations, have been already used for single-cell oil production by strains of *Y. lipolytica*

400 grown on various agro-industrial by-products or waste, which could be perfect sources for sole

401 CLA isomer manufacture.

402 **5.3 Oleaginous fungus**

403 An oleaginous fungus, *Mortierella alpina*, has been used for commercial production of

404 arachidonic acid (AA), in which fatty acids could accumulate up to approximately 50% of DCW.

405 The relative abundance and proportion of its PUFAs makes *M. alpina* a perfect source of some

406 specific nutritional supplements. Moreover, the genome of *M. alpina* has been fully

407 characterized [114] and an *Agrobacterium tumefaciens*-mediated transformation (ATMT)

408 system has been established successfully [115]. As *M. alpina* could accumulate a high content

409 of PUFAs, including linoleic acid (~10% of total fatty acids), it could be a valuable organism for

410 CLA production. The incorporation of *t*10,*c*12 CLA into the PUFAs of *M. alpina* via PAI

411 conversion, if achieved at a high level, would significantly increase the commercial value of *M.*

412 *alpina*. We tried heterologously expression of codon-optimized PAI gene in *M. alpina* via ATMA

413 system [116]. The amount of *t*10,*c*12-CLA increased up to 1.2 mg/l and reached up to 29 mg/l

414 when acyl CoA synthetase inhibitor was added. With free LA and Triacsin C addition together,

415 the final CLA reached 4.05% TFA (Fig 7). Heterologous expression of PAI in *M. alpina* could be

416 considered as another practicable way for industrial production of CLA.

6. Perspectives

6.1 Further mechanistic exploration for lactic acid bacteria.

419 Recently, the system control of CLA production among lactic acid bacteria could be

420 divided into two groups, group I and group II. Group I includes three genes that is *mcra, cla-dh*

421 *and cla-dc* and the synthesis pathway has been elucidated. Group II (mainly among bacteria

422 strains) has been identified to be a new system for CLA production, in which

423 10-hydroxy-octadecenic acid is not one of the intermediates, but the intricate mechanism is not

424 clear. Thus, more efforts should focus on exploring this new system, for the purpose of

425 broadening our knowledge about CLA production among lactic acid bacteria and providing

426 more synthetic routes of CLA for manufacturers.

6.2 Microbial cell factories development and manufacturing of CLA.

428 Commercial CLA are produced through chemical process or extract from plant seeds,

429 which result in mixtures of CLA in the final products. A number of bacteria have been reported

430 to produce unique CLA effectively, which could be potential microbial cell factories for CLA

431 production, whereas fermentation cost and low concentration of CLA, those high CLA

432 producers might not be ideal microbial cell factories. Recombinant technology seems to be an

433 effective alternative. For oleaginous microorganisms, *Y. Lipolytica* and *M. alpina*, have been

434 successfully introduced for 10,12-CLA production, which is much close to industry purpose.

435 Later, fermentation condition should be optimized to meet the industrial needs. The main

436 9,11-CLA producers include *B. fibrisolvens*, *C. sporogenes, L. plantarum, Bifidobacterium.*

437 Among all these strains, only the genes among *L. plantarum* responsible for the CLA

438 production were clear, including *mcra*, *cla-dh* and *cla-dc*. In the future, many trials should be

439 carried out to express these three genes in some model engineering strains, such as *Y.*

440 *lipolytica* and *M. alpina.*

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Figure captions

Fig. 1 Proposed model for the isomerization of linoleic acid by linoleic acid isomerase in rumen microorganisms.

An electrophile (E), at the active site, interacts with one of the substrate double bonds and two basic centers also interact with the substrate. One of the basic centers (B) is hydrogen bonded to the carboxyl group of the substrate and the other (B-H) serves as the donor of the hydrogen added at C-13 (From Kepler et al., 1971).

Fig. 2 Different conformation of domain 4 observed in MCRA monomers from *L. acidophilus*.

(A) A superposition of two MCRA protomers differing in the conformation of domain 4. Domain 1, 2, 3 and 4 of the apo MCRA protomer (symmetric protomer) are coloured marine, light green, red and yellow, respectively. The LA-MCRA protomer (asymmetric protomer) exhibiting a different conformation of domain 4 is coloured grey. The missing residues of domain 4 (556-574) are marked as a grey dashed line and the remaining traceable fragment of domain 4. (B) Superposition of apo LAH and LA–LAH dimers viewed along the dimerization axis. Domains 1, 2, 3 and 4 of apo LAH are coloured marine, light green, red and yellow/wheat, respectively. The protomomers of LA–LAH are coloured in grey and dark grey for the symmetric and the asymmetric protomer, respectively. Residue Lys575 is labelled in both the symmetric protomer (apo LAH, loop region) and the asymmetric protomer. (C) Linoleic acid bound at the entrance to the MCRA substrate and the electron density observed at the substrate-entrance channel. (D) Linoleic acid bound at the entrance to the LAH substrate channel. (From Volkov et al., 2013)

Fig. 3 CLA production pathway and hydroxyl fatty acid generation in lactobacilli

10-HOE was one of the intermediates during CLA production in *L. plantarum* and *L. acidophilus*. 10-LAH, DH and DC were the enzymes for CLA production in *L. plantarum* AKU1009a and *L. plantarum* ZS2058, while 10-LAH and enolase were the enzymes for CLA production in *L. plantarum* ATCC 8014. 10-LAH: linoleate 10-hydratase, 13-LAH: linoleate 13-hydratase, DH: short-chain dehydrogenase/oxidoreductase; DC: acetoacetate decarboxylase. 13-HOE: 13-hydroxy-*cis*-9-octadecenoic acid; 10-HOE:

10-hydroxy-*cis*-12-octadecenoic acid; 10,13-diHOA: 10,13-hydroxy-octadecanoic acid.

Fig. 4 Structure-based isomerization mechanism of linoleic acid to 10, 12-CLA

(A) Architecture of PAI. The FAD-binding domain 1 is colored in magenta, domain 2 in red, and domain 3 in blue. FAD and polyethylene glycerol (PEG) 400 are shown as stick models. (B) The surface potential of PAI. (C) The molecular surface (blue) of part of the PEG400 molecule bound to PAI in absence of substrate/product. (D) Conformational changes in active site associated with PEG400 binding reveal the gating mechanism. (From Liavonchanka et al., 2006). (E) Structure-based isomerization mechanism of linoleic acid to *t*10,*c*12-CLA

Fig. 5 CLA production strategy in *Yarrowia lipolytica* and in *Mortierella alpina*

DHAP: dihydroxyacetone phosphate; G3P: glycerol-3-phosphate; LPA: lysophosphatidic acid PA: phosphatidic acid; DAG: diacylglycerol; TAG: triacylglycerol; FFA: free fatty acids.

G3PD: G3P dehydrogenase; *SCT*: glycerol-3-phosphate acyltransferase; *PAP*: PA phosphohydrolase; *DGA*: acyl-CoA:DAG acyltransferase; *TGL*: triacylglycerol lipase; *ACOT*: Acyl-CoA thioesterase; *FAA*: fatty acyl-CoA synthetase; *FAS*: fatty acid synthase; *PAI*: polyunsaturated fatty acid isomerase. (Those genes and approach for enhancing CLA production were highlighted in red arrows and cycles, in which the solid ones have been done. Those genes and pathway for reducing CLA accumulation were highlighted in green arrows.)
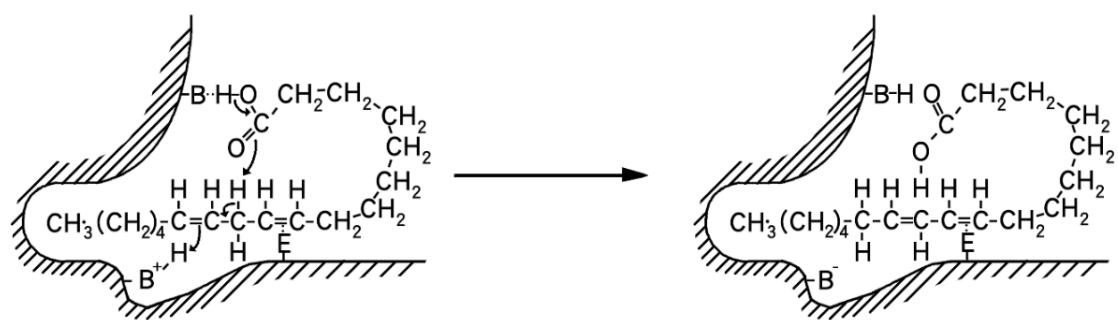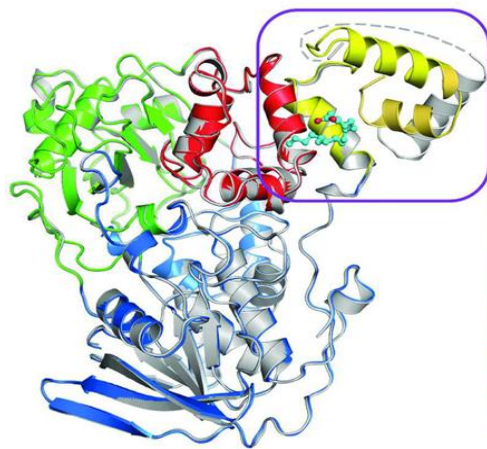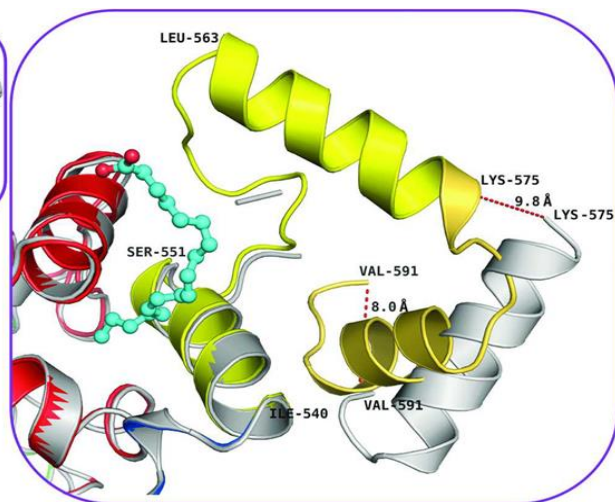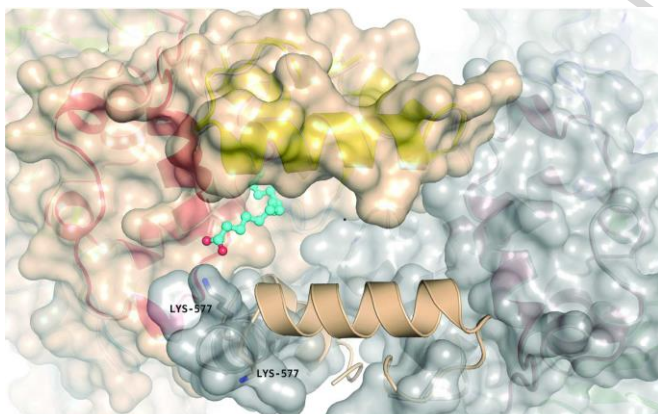
Fig. 1

Fig. 2

(A)

(B)

(C)

(D)

Fig. 3

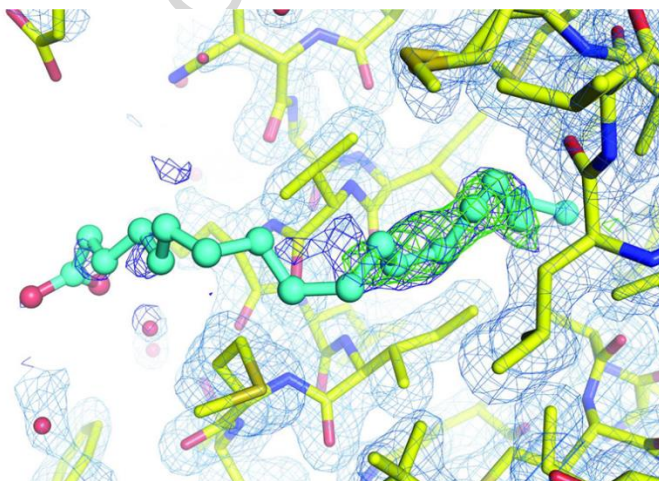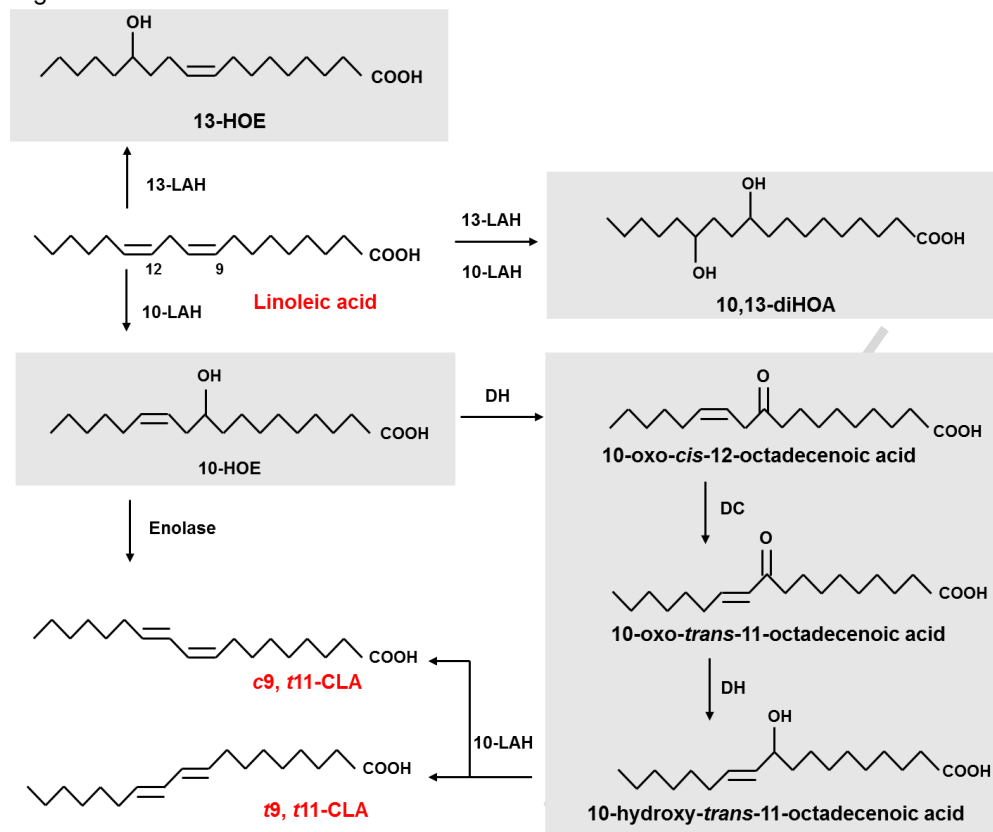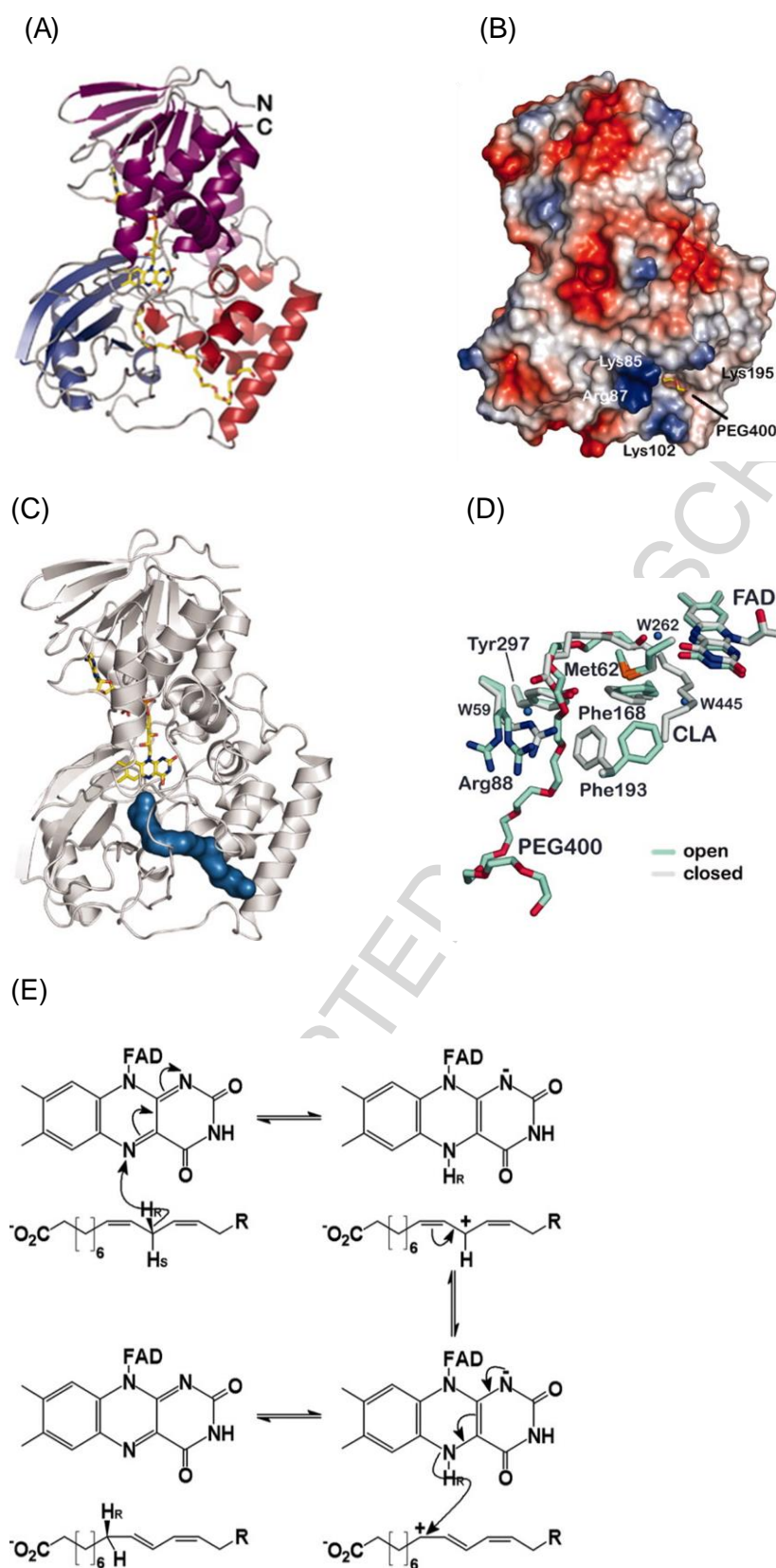Fig. 4

(A)

(B)

(C)

(D)

(E)

Fig. 5

Table 1 High CLA-producing strains

Species	Strain No.	Substrate (g/l)	Catalyst	Total CLA (g/l)	Conversion (%)	9,11-CLA (%)	10,12-CLA (%)	Ref.
Butyrivibrio fibrisolvens	A38	LA (0.10)	washed cells	0.080	80.0	95	5	[37]
Lactobacillus acidophilus	1.184	LA (2.00)	washed cells	1.728	86.4			[117]
	CRL 730	LA (0.20)	growing cells	0.048	23.8			[71]
	Q42	LA (0.20)	growing cells	0.040	20.0			[71]
	L1	LA (0.20)	growing cells	0.131	65.5	90	10	[21]
	O16	LA (0.20)	growing cells	0.061	30.5	91	9	[21]
	AKU 1137	LA (4.00)	washed cells	1.500	37.5	100	0	[19]
	AKU 1137	LA (5.00)	growing cells	4.900	98.0	100	0	[28]
Lactobacillus brevis	IAM 1082	LA (4.00)	washed cells	0.550	13.8	100	0	[19]
Lactobacillus casei	CRL 431	LA (0.20)	growing cells	0.072	35.9			[71]
	E10	LA (0.20)	growing cells	0.080	40.1	91	9	[21]
	E5	LA (0.20)	growing cells	0.111	55.5	88	12	[21]
Lactobacillus plantarum	JCM 1551	LA (4.00)	washed cells	2.020	50.5	100	0	[19]
	JCM 1551	castor oil (5.00)	growing cells	2.700	54.0	100	0	[44]
	ZS2058	LA (0.55)	growing cells	0.300	54.5	56	3	[45]
	lp 15	LA (0.10)	growing cells	0.026	26.1	76	24	[118]
	PL62	LA (1.00)	crude enzyme	2.65*		47	53	[119]
	NCUL005	LA (2.28)	growing cells	0.602	26.4	32	69	[82]
Lactobacillus pentosus	C14	LA (0.20)	growing cells	0.069	34.5			[71]
Lactobacillus reuteri	ATCC55739	LA (0.55)	growing cells	0.350	63.6	97	3	[45]
	ATCC55739	LA (0.90)	growing cells	0.300	33.3	59	41	[18]
Bifidobacterium animalis	Bb12	LA (0.56)	growing cells	0.170	30.4	31	1	[45]
Bifidobacterium breve	NCFB2257	LA (0.55)	growing cells	0.231	42.0	99	1	[65]
	NCFB 2258	LA (0.55)	growing cells	0.398	72.4	100	0	[65]
	NCFB11815	LA (0.55)	growing cells	0.215	39.1	99	1	[65]
	NCFB8815	LA (0.55)	growing cells	0.242	44.0	99	1	[65]
	NCFB 8807	LA (0.55)	growing cells	0.128	23.3	99	1	[65]
	LMC 520	LA (0.56)	growing cells	0.400	71.4	95	5	[23]
Bifidobacterium bifidum	CRL 1399	LA (0.20)	growing cells	0.050	24.8			[71]
Bifidobacterium lactis	Bb12	LA (0.55)	growing cells	0.170	30.9	98	2	[65]
Enterococcus faecium	M74	soy oil (10) [b]	growing cells	0.73[a]		100	0	[77]
Megasphaera elsdenii	YJ-4	LA (0.02)	growing cells	0.007*		15	85	[80]
Pediococcus acidilactici	AKU1059	LA (4.00)	washed cells	1.400	35.0	100	0	[19]
Propinibacterium acnes	No.27	LA (0.02)	growing cells	0.017	85.0	0	100	[26]
Propinibacterium	P-6 Wiesby	LA (0.75)	growing cells	0.265	35.3	93		[73]

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freudenreichii	9093	LA (0.50)	growing cells	0.111	22.2	90		[73]
	ATCC 6207	LA (0.10)	growing cells	0.023	23.2	75		[73]
Propinibacterium	56	soy oil (10) [b]	growing cells	1.09[a]		83	17	[77]
freudenreichii subsp.	51	soy oil (10) [b]	growing cells	1.65[a]		85	15	[77]
shermanii	23	soy oil (10) [b]	growing cells	0.81[a]		75	25	[77]
Streptreptococcus	CRL 728	LA (0.20)	growing cells	0.068	33.9			[71]
thermophilus	CRL728	LA (0.20)	growing cells*	0.105	52.5			[71]

* represents the reaction occurs in the skim milk;
[a] represent the results is expressed as mg CLA content per gram lipid; [b] means that soy oil is used in the hydrolyzed form.