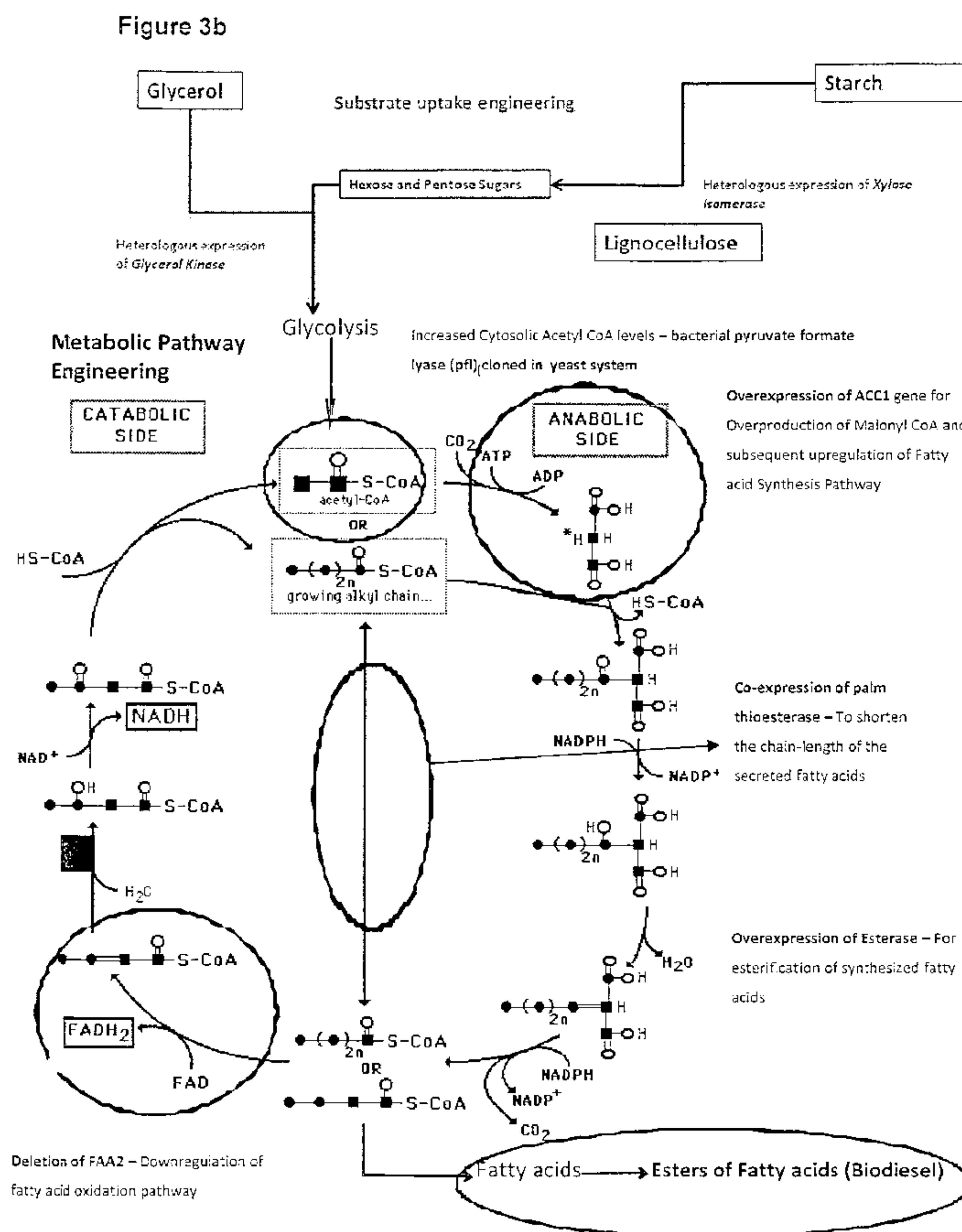




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 (71) Demandeur/Applicant:
 TECHNICAL UNIVERSITY OF DENMARK, DK
 (72) Inventeurs/Inventors:
 PHADNAVIS, AMBAREESH GOVIND, DK;
 RUHDAL JENSEN, PETER, DK
 (74) Agent: GOWLING LAFLEUR HENDERSON LLP

(54) Titre : PRODUCTION DE BIODIESEL PAR UNE LEVURE A PARTIR DE LIGNOCELLULOSE ET DE GLYCEROL
 (54) Title: PRODUCTION OF BIODIESEL BY YEAST FROM LIGNOCELLULOSE AND GLYCEROL



(57) Abrégé/Abstract:

The invention is directed to a genetically modified microorganism for the extracellular production of free fatty acids and esters thereof, wherein said microorganism is characterised by a modified lipid biosynthesis metabolic pathway: for example reduced fatty

(57) **Abrégé(suite)/Abstract(continued):**

acyl-coA synthetase activity that enables the microorganism to overproduce and secrete of esters of fatty acids (Biodiesel) into the surrounding medium, using one or more of: glucose, starch, lignocellulose and a glycerol-based substrate, as a carbon source. The invention further provides a method for the extracellular production of free fatty acids and esters thereof, comprising the use of said genetically modified organism, and a growth medium adapted for said method.

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(72) Inventor; and

(75) Inventor/Applicant (for US only): PHADNAVIS, Am-
bareesh Govind [IN/DK]; Electrovej 330, P-2, DK-2800
Kgs. Lyngby (DK).

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(74) Agent: NILAUSEN, Kim; Zacco Denmark A/S, Hans
Bekkevolds Allé 7, DK-2900 Hellerup (DK).

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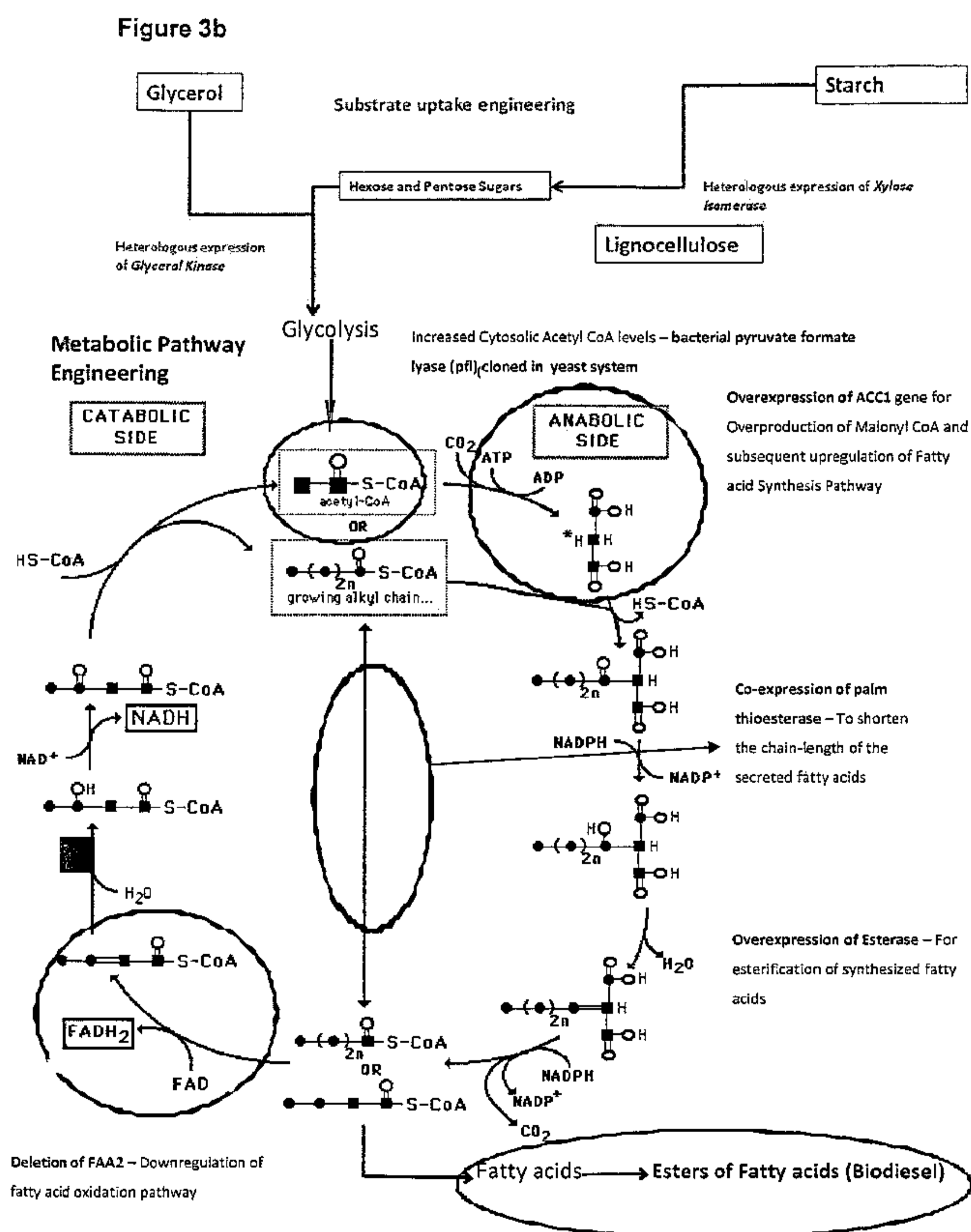
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(71) Applicants (for all designated States except US): **TECH-**
NICAL UNVIERSITY OF DENMARK [DK/DK];
Anker Engelunds Vej 1, DK-2800 Kgs. Lyngby (DK).
RUHDAL JENSEN, Peter [DK/DK]; Søgardsvej 19,
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(54) Title: PRODUCTION OF BIODIESEL BY YEAST FROM LIGNOCELLULOSE AND GLYCEROL



(57) Abstract: The invention is directed to a genetically modified microorganism for the extracellular production of free fatty acids and esters thereof, wherein said microorganism is characterised by a modified lipid biosynthesis metabolic pathway: for example reduced fatty acyl-coA synthetase activity that enables the microorganism to overproduce and secrete of esters of fatty acids (Biodiesel) into the surrounding medium, using one or more of: glucose, starch, lignocellulose and a glycerol-based substrate, as a carbon source. The invention further provides a method for the extracellular production of free fatty acids and esters thereof, comprising the use of said genetically modified organism, and a growth medium adapted for said method.

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Title: Production of Biodiesel by Yeast from Lignocellulose and Glycerol

Field of the invention

- 5 The invention relates to modifying the lipid biosynthesis metabolic pathway in yeast by genetic engineering in order to overproduce and secrete esters of fatty acids (Biodiesel) into the medium, using one or more of: glucose, starch, lignocellulose and a glycerol-based substrate, as a carbon source.

10 Background of the invention

- Biodiesel refers to a fuel comprised of mono-alkyl esters of long chain fatty acids derived from vegetable oils or animal fats, designated B100, and meeting the requirements of ASTM D 6751. Currently, the most common method of Biodiesel production is trans-esterification of edible and non-edible
15 vegetable oils, or sometimes animal fats. The trans-esterification reaction transforms triglycerides into fatty acid alkyl ester, in the presence of an alcohol, such as methanol or ethanol, and a catalyst, such as an alkali or acid, where glycerol is a by-product. Biodiesel has a comparable cetane rating and energy content to petro-diesel and hence is considered to be a
20 perfect substitute to petro-diesel. It can either be used in pure form (100%) or is blended with petro-diesel in varying proportions. Soybean and Rapeseed are a common source of vegetable oils for biodiesel production. Such vegetable oils are in the form of triglycerides, which then need to be degraded to free-fatty acids and glycerol, where the fatty acids are esterified
25 either chemically or enzymatically by the process known as 'transesterification'. This poses an enormous strain on available arable land for production of both food-crops and fuel-crops, thereby exerting an undesirable pressure on food prices. Furthermore, since oil-seeds are a seasonal crop, once the crop is harvested and utilized, one has to wait until
30 the next harvest is ready. Accordingly, there exists a need to overcome the food-OR-fuel dilemma, as well as a continuing need to shift global energy

consumption from fossil fuels to environment-friendly biofuels. A further goal is to exploit energy sources such as cheap carbon sources which are by-products of established biodiesel Industry (Glycerol), as well as the most abundant non-edible sugar-sources available in nature, namely lignocellulose. The use of non-starch carbon sources such as cellulose, hemicelluloses, xylose, and lignin will not only favour the economics of bulk production of biodiesel, but will also reduce the strain on agricultural resources needed to satisfy society's need for food.

Although attempts have been made to manipulate microbial organisms biodiesel production, a principal barrier to success has been the development of robust, high-yielding microbes and processes for their production

Summary of the invention

The present invention is directed to a genetically modified microorganism for the extracellular production of free fatty acids and esters thereof, wherein said microorganism characterised by: reduced fatty acyl-coA synthetase activity conferred by one or more of: FAA2 gene deletion; deletion of any one of a FAA1 gene, FAA3 gene and FAA4 gene; recombinant endogenous FAA 1 gene, FAA3 gene or FAA4 gene, wherein said gene is operably linked to a heterologous promoter; and/or enhanced acetyl CoA carboxylase expression conferred by: a recombinant endogenous ACC1 gene wherein said gene is operably linked to a heterologous promoter. For example, said microorganism is selected from among species of *Aspergillus*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Fusarium*, *Lindnera*, *Lipomyces*, *Monascus*, *Mucor*, *Pachysolen*, *Pichia*; *Rhizopus*, *Rhodosporidium*; *Rhodotorula*, *Saccharomyces*; *Schizosacchomyces*; *Trichosporon*, *Yarrowia*, and *Zygosacchomyces*. Preferably the genetically modified organism is a *Saccharomyces*, in particular *S. cerevisiae*.

In one embodiment the genetically modified microorganism is characterised by a recombinant endogenous ACC1 gene that is operably linked to a TEF1 promoter from yeast [SEQ ID NO: 113].

5 In one embodiment the genetically modified microorganism is characterised by comprising a transgene encoding a pyruvate formate lyase comprising PflA and PflB.

The genetically modified microorganism comprising either a FAA2 deletion (or a FAA1, FAA3 or FAA4 deletion), or a recombinant endogenous ACC1 gene that is operably linked to a heterologous promoter, may be
10 characterised by additionally comprising the transgene encoding a pyruvate formate lyase comprising PflA and PflB.

The genetically modified microorganism may be further characterised by additionally comprising a transgene encoding an acyl CoA-ACP thioesterase, wherein said thioesterase is selected from among: Soyabean (*Glycine max*);
15 *Chlamydomonas reinhardtii* (Protein ID - A8HY17); *Arabidopsis thaliana* (Protein ID - Q9SJE2); *Ricinus communis* (Protein ID - B9RAC3); *Triticum aestivum*; CtFatA from *Brassica napus* (Protein ID - Q43745); CtFatA from *C. tinctorius* (Protein ID - Q42715); GmFatA1 from *G. mangostana* (Protein ID - O04792); CwFatB1 from *C. hookeriana* (Protein ID - Q39513); CwFatB1 from
20 *C. wrightii* (Protein ID - Q39662); GmFatB1 from *G. Mangostana* (Protein ID - O04794).

The genetically modified microorganism, may be further characterised by additionally comprising enhanced expression of an acyl-coenzymeA:ethanol O-acyltransferase conferred by a recombinant endogenous acyl-coenzymeA:ethanol O-acyltransferase (EEB1) gene wherein said gene is
25 operably linked to a heterologous promoter.

The genetically modified microorganism may be further characterised by additionally comprising a transgene encoding a heterologous cytosolic Acyl

CoA thioesterase (CTE-1), wherein said CTE-1 is selected from among: *Mus musculus* (Protein ID - O55137); *Arabidopsis thaliana* (Protein ID - Q5FYU1) or *Rattus norvegicus* (Protein ID - Q6AZ44)

5 The genetically modified microorganism may be further characterised by additionally comprising one or more transgene encoding a heterologous glycerol kinase or a xylose isomerase or both; wherein said glycerol kinase is selected from among Glycerol Kinase from *Saccharomyces cerevisiae* (Protein ID - P32190), and said xylose isomerase is selected from among Xylose isomerase from *Clostridium phytofermentas* (Protein ID - A9KN98);
10 *Yersinia pestis* – (Protein ID - Q8Z9Z1).

The genetically modified microorganism may be further characterised by additionally comprising a deletion of an endogenous formate dehydrogenase FDH gene wherein said FDH gene is selected from among: GeneID: 854570; GeneID: 8300341;and GeneID: 2907923.

15 The genetically modified microorganism may be further characterised by additionally comprising a transgene encoding a heterologous formate hydrogen lyase, where said lyase is *E.coli* formate hydrogen lyase (Protein ID - C8UET5)

20 The genetically modified microorganism may be further characterised by additionally comprising a deletion of an endogenous alcohol dehydrogenase (ADH) gene, wherein said ADH gene is selected from among: GeneID: 854068; GeneID: 2538902; GeneID: 2868277; and GeneID: 852442.

The present invention is also directed to the use of a yeast or fungal strain for the extracellular production of free fatty acids and esters thereof, wherein
25 said yeast or fungal strain is selected from among: *Candida tropicalis*, *Pachysolen tannophilus*, a FAA2 deletion strain of yeast, and a yeast or fungal strain according to any one of the above described genetically modified microorganism.

The present invention is also directed to a method for the extracellular production of free fatty acids and esters thereof, comprising the steps of: a) introducing a microorganism yeast or fungal strain into a growth medium to produce a culture; b) incubating said culture, wherein the growth medium in said culture is aerated with oxygen; c) further incubating said culture after step (b) in the absence of an oxygen supply; d) recovering an extracellular phase comprising free fatty acids and esters from said culture; wherein said microorganism is a yeast or fungal strain.

The method may further use a microorganism selected from among *Candida tropicalis*, *Pachysolen tannophilus* or any of the above described genetically modified microorganism.

In one embodiment the growth medium of the method comprises a carbon source is selected from at least one of glucose, glycerol, xylose, hydrolysed cellulose and hemicellulose, starch, sugar alcohol and xylan, Preferably the growth medium comprises or consists of the components set out below.

The present invention is also provides a growth medium adapted for use in the above described method, wherein the medium the composition comprises the following components,

Component	Composition (grams/litre)
Carbon Source	
carbon source	≥50 g
Nitrogen Source	
Ammonium sulphate	5g
Vitamins*	
Biotin	20 µg
Folic acid	2 µg
Inositol	10 mg
Niacin	400 µg
Riboflavin	200 µg
Pyridoxine hydrochloride	400 µg
Thiamine hydrochloride	400 µg
Compounds supplying trace elements	

Boric acid	500 µg
Copper sulphate	40 µg
Potassium iodide	100 µg
Ferric chloride	200 µg
Manganese sulphate	400 µg
Sodium molybdate	200 µg
Zinc sulphate	400 µg
Salts	
Potassium phosphate monobasic	900 mg
Potassium phosphate dibasic	200 mg
Magnesium sulphate	500 mg
Sodium chloride	100 mg
Calcium chloride	100 mg

and wherein said carbon source is selected from among glucose, glycerol, xylose, hydrolysed cellulose and hemicellulose, starch, sugar alcohol and xylan.

5 Detailed description of the invention

Figures:

Figure 1a: Saponifiable fatty acid content of two yeast strains (Y axis) as a function of fermentation growth temperature (X-axis). Note that the values of control reading are on X axis with Y co-ordinate as zero throughout the experiment.

Figure 1b: Saponifiable fatty acid content of two yeast strains (Y axis) as a function of glucose concentration (X-axis). The relationship is linear passing through origin, however, due to practical difficulties, the trend was discontinued beyond X=20.

Figure 1c: Saponifiable fatty acid content of the fermentation medium of two yeast strains (Y axis) as a function of pH values (X-axis). The trend is roughly a Bell Shaped curve

Figure 2: Gas Chromatogram of methyl esters of fatty acids secreted into the medium by yeast strain *Saccharomyces cerevisiae* (Wild type).

Figure 3: Lipid metabolic cycle in yeast. **a)** FAA2 is one of the enzymes in the repertoire of *Saccharomyces cerevisiae* which catalyzes the first step involved in catabolism of fatty acids; **b)** manipulation of the lipid metabolism in yeast.

5 **Figure 4:** The deleted portion of FAA2. 145bp upstream and 60bp downstream of the gene were deleted and replaced with URA3

Figure 5: SDS-gel with PCR amplification products of FAA2 from WT genome. Lane 1: DNA size marker:100 – 10,000 nucleotide base pairs; Lane 2: PCR product of WT yeast genomic DNA amplification using primer pair 3
10 (Table 4).

Figure 6a,b: Detection of FAA2 (Δ ura3) deletion in gDNA from transformed yeast colonies. Wells 3-9, 11-17 contain genomic DNA from transformed yeast colonies, amplified with FAA2 primer set 3. Wells 18, 20-27, 30-33 contain genomic DNA from transformed yeast colonies, amplified with URA3
15 primer set 2. Wells 1, 10, 19, 28, 29, 40 contain DNA size ladders. Well number 2 and 39 contained WT-DNA amplified with primer set 3 and primer set 2 respectively. Well 38 contain URA3 plasmid amplified with primer set 2, as positive control.

Figure 7: GC-MS graph of extracellular fatty acids secreted by from WT-strain (*S. cerevisiae*) grown on glucose medium.
20

Figure 8: GC-MS graph of intracellular fatty acids secreted by from WT-strain (*S. cerevisiae*) grown on glucose medium.

Figure 9: GC-MS graph of extracellular fatty acids by FAA2 (Δ ura3) deletion mutant of WT-strain (*S. cerevisiae* FAA2 Δ ura3) grown on glucose medium.

25 **Figure 10:** GC-MS graph of intracellular fatty acids by FAA2 (Δ ura3) deletion mutant of WT-strain (*S. cerevisiae* FAA2 Δ ura3) grown on glucose medium.

Figure 11: GC-MS graph of extracellular fatty acids secreted by from WT-strain (*S. cerevisiae*) grown on glycerol medium.

30 **Figure 12:** GC-MS graph of extracellular fatty acids by FAA2 (Δ ura3) deletion mutant of WT-strain (*S. cerevisiae* FAA2 Δ ura3) grown on glycerol medium.

Figure 13: GC-MS graph of extracellular fatty acids by *Candida tropicalis* grown on glucose medium (upper panel); fatty acid standards (lower panel).

Figure 14: GC-MS graph of extracellular fatty acids by *Candida tropicalis* grown on glycerol medium (upper panel); fatty acid standards (lower panel).

5 **Figure 15:** The homologous recombination of upstream and downstream sequences from the bipartite gene-targeting substrate to the chromosomal locus results in the exchange of *ACC1* promoter to *TEF1* promoter and the insertion of *KI URA3* flanked by direct repeats (DR). The *KI URA3* was later removed by plating the strains on medium containing 5-fluoroorotic acid (5-
10 FOA).

Figure 16: PCR detection of *FAA2* (Δ ura3) deletion in gDNA from transformed SC-*ACC1* yeast colonies. Gel in which lanes 1-4 contain genomic DNA from transformed yeast colonies, amplified with *FAA2* primer set 3 and *URA3* primers respectively, and demonstrate presence of *URA3*
15 and absence of *FAA2* in the gDNA in mutants in Lanes 1, 2 and 4. Lane 5 contains a DNA size ladder.

Figure 17: PCR detection of *E.coli* pyruvate formate lyase A gene (in lane 1 and 4) and Pyruvate Formate lyase B gene (in lane 2 and 3) amplified from *E.coli* genome, amplified with *pflA* and *pflB* specific primers. Lane M contains
20 a DNA size ladder.

Figure 18: Gel Photograph showing plasmid (in lane 2 and 3) and ligated plasmid (in lane 1 and 4). The lane 1 is *PflA* gene in shuttle vector PCM182. The lane 4 is *PFLB* gene in shuttle vector PCM183. Lane M contains a DNA size ladder.

25 **Figure 19:** Comparative fatty acid yield of yeast strains of invention when grown on 20% glucose as carbon source.

Figure 20: Comparative fatty acid yield of yeast strains of invention when grown 5% pure glycerol as carbon source.

30 **Figure 21:** Comparative fatty acid yield of yeast strains of invention when grown on 5% crude glycerol as carbon source.

Figure 22: Comparative fatty acid yield of yeast strains of invention when grown on 15% xylose as carbon source

Figure 23: Comparative fatty acid yield of yeast strains of invention when grown on 10ml/L hydrolysed wheat lignocellulose as carbon source

5

Aim of the invention:

The present invention provides an alternative source of biodiesel, which relies on modified yeast strain(s), whose metabolic pathway for Lipid Biosynthesis is specifically engineered to overproduce and secrete esterified fatty acids when supplied with one or more substrates such as glucose, starch, glycerol and lignocellulose. While the fatty acids and their esters that are secreted by the yeast strain(s) commonly vary in chain-length, wherein a high proportion of the fatty-acids produced are long-chain fatty acids (C:16, C:18 and longer), it is desirable to produce fatty acids and their esters of slightly shorter chain-lengths, since these are considered to be qualitatively superior as a source of biodiesel.

10
15

Definitions:

ACC1 – gene encoding acetyl CoA carboxylase.

FAA2 – gene encoding long chain fatty acyl-CoA synthetase (Faa2p; EC No: 6.2.1.3) that accepts a wider range of acyl chain lengths than Faa1p, preferring C9:0-C13:0; and is involved in the activation of endogenous pools of fatty acids;

MCFA – medium chain fatty acid

pflA – gene encoding pyruvate formate lyase A (Pfl A)

pflB - gene encoding pyruvate formate lyase B (PflB)

SCFA – short chain fatty acid

VLCFA – very long chain fatty acid

30 **1.0 Selection of the microorganism of the invention**

The microorganism of the invention is a yeast or fungal species, since yeast and fungi can accumulate oils under some cultivation conditions, and some yeast and fungi species secrete fatty acids into the medium when grown on certain carbon sources. Accordingly, a yeast or fungal species of the invention is one that is able to secrete fatty acids extracellularly in the medium, and is capable of producing a large cell biomass combined with and a high extracellular lipid yield. A preferred yeast or fungal species according to the invention is a yeast or fungal species capable of secreting fatty acids and esters thereof, said yeast or fungal species belonging to the genus *Aspergillus* (e.g. *A. nidulans*), *Candida* (e.g. *C. tropicalis*; *C. magnolia*); *Cryptococcus* (e.g. *C. albidus*), *Debaryomyces* (e.g. *D. hansenii*), *Fusarium* (e.g. *F. oxysporum*), *Lindnera* (e.g. *L. jadinii*), *Lipomyces* (e.g. *L. lipofera* or *L. starkeyi*), *Monascus* (e.g. *M. purpureus*), *Mucor* (e.g.; *M. circinelloides*, *M. hiemalis*; *M. miehei*; *M. racemosus*), *Pachysolen* (e.g. *P. tannophilus*), *Pichia* (e.g. *P. pastoris*; *P. stipitis*; *P. angusta*); *Rhizopus* (e.g. *R. oryzae*), *Rhodosporidium* (e.g. *R. toruloides*); *Rhodotorula* (e.g. *R. glutinis*), *Saccharomyces* (e.g. *S. cerevisiae*); *Schizosacchomyces* (e.g. *S. pombe*); *Trichosporon* (e.g. *T. pullulan*), *Yarrowia* (e.g. *Y. lipolytica*), *Zygosacchomyces* (e.g. *Z. rouxii*), or a strain of any one thereof. Preferably the micro-organism is a *Saccharomyces*, in particular *S. cerevisiae*.

In one embodiment of the invention, the microorganism belongs to the yeast genus *Candida*, in particular the species *Candida tropicalis*, said yeast being characterised by the secretion of palmitic acid.

2.0 Genetically modified yeast or fungal species of the invention, adapted to synthesize and secrete MCFAs

A genetically modified yeast or fungal species/strain of the invention is preferably derived from a microorganism selected from the group set out above under section I. One or more gene controlling the metabolic pathways in the selected yeast or fungal species/strain is genetically modified to enable

the selected species/strain to produce and secrete esters of fatty acids from various cheap carbon sources such as starch, glycerol and lignocellulose.

2.1.0 A genetically modified yeast or fungal strain with reduced fatty acid catabolism

2.1.1 Deletion of endogenous fatty acyl-CoA synthetase gene, FAA2

In one embodiment, a yeast or fungal species/strain of the invention carries a deletion in the FAA2 gene (FAA2 Δ strain) encoding the Faa2p, which catalyses the activation of medium-chain fatty acids, being the first committed step in beta-oxidation of these fatty acids. Deletion of FAA2 gene, in the yeast or fungal species, reduces the metabolic flux through fatty acid catabolism. In addition a FAA2 Δ yeast or fungal species of the invention is characterised, not only by the synthesis of a surprisingly higher proportion of MCFAs than wild type yeast, but as further being capable of extracellular secretion of the MCFAs synthesized within the cell. Where the yeast strain is *Saccharomyces cerevisiae*, the deleted FAA2 gene is Gene ID: 856734 (SEQ ID No: 1) and encodes FAA2 (EC 6.2.1.3) having protein ID: P39518 (SEQ ID No: 2).

2.1.2 Silencing endogenous fatty acyl CoA synthetase gene expression

In a further embodiment, expression of the one or more endogenous fatty acyl-CoA synthetase (FAA) genes in the yeast or fungal species of the invention is silenced or knocked-down by means of gene deletion or by means of promoter engineering. For example, replacement of the native FAA gene promoters with promoters driving lower expression levels is used to drastically reduce the expression levels of gene FAA genes and their encoded enzymes located in peroxisomes and mitochondria.

Thus the promoters of each of the *S.cerevisiae*, FAA1, FAA3, FAA4 may be substituted with promoters driving lower expression levels. A corresponding strategy may be applied to silence or knock-down the expression of FAA

genes in other yeast species. In one example the FAA gene to be silenced by gene deletion or knocked out by means of promoter engineering is one or more of the Faa1 gene (GeneID: 854495 (SEQ ID No: 3); encoding Protein ID - P30624: (SEQ ID No: 4)); the Faa3 gene (GeneID: 854808 (SEQ ID No: 5) encoding Protein ID - P39002 (SEQ ID No: 6)), and the Faa4 (GeneID: 855288 (SEQ ID No: 7) encoding Protein ID - P47912 (SEQ ID No: 8)) in *Saccharomyces cerevisiae*; the FAA gene (GeneID: 2541350 (SEQ ID No: 9) encoding Protein ID - Q9P7D7 (SEQ ID No: 10)) in *Schizosaccharomyces Pombe*; the FAA gene (GeneID: 3257561 (SEQ ID No: 11) encoding Protein ID - Q5KH65 (SEQ ID No: 12)) in *Cryptococcus neoformans*; the FAA gene (GeneID: 8300178 (SEQ ID No: 13) encoding Protein ID - C5MID6 (SEQ ID No: 14)) in *Candida tropicalis*; the FAA gene (GeneID: 2876383 (SEQ ID No: 15) encoding Protein ID - Q5BFS3 (SEQ ID No: 16)) in *Aspergillus nidulans*; and the FAA gene (GeneID: 2911089 (SEQ ID No: 17) encoding Protein ID - Q6C8Q3 (SEQ ID No: 18)) in *Yarrowia lipolytica*.

2.1.3 Inhibition of fatty acyl-CoA synthetase activity

In a further embodiment, the enzymatic activity of the endogenous fatty acyl CoA synthetase (FAA), in the yeast or fungal species of the invention, can be inhibited by means of inhibitors, for example with triacsin C (Pubchem. ID - CID: 9576787) or adenosine 5'-hexadecylphosphate. Triacsin-C can be added to the growth medium to inhibit FAA activity and thereby increase the levels of secreted fatty acids in the medium. This approach is useful in those cases where the FAA gene in the respective yeast or fungal species cannot be genetically modified.

2.1.4. Disruption and inhibition of FATp1

In a further embodiment, the gene encoding the fatty acid transporter, FATp1 in the yeast or fungal species of the invention, is disrupted or inhibited. Lipid metabolism is compartmentalized in *Saccharomyces cerevisiae*, whereby the biosynthetic enzymes are located in the cytosol of the cell, whereas the

catabolic enzymes are located in peroxisomes and mitochondria. In order for fatty acids to be oxidized, they must be transported into these respective cellular organelles by the means of transporter protein like "Fatty acid transporter" (FAT1), located on peroxisomal membrane. Fat1 is a medium chain fatty-acid-CoA activase itself. Thus, inhibition and/or disruption of this enzyme prevents the entry of fatty acids into peroxisomes, thereby reducing metabolic flux through the lipid catabolic pathway. In one example the FATp1 gene to be disrupted or inhibited FAA gene is the fatty acid transporter gene (GeneID: 852329 (SEQ ID No: 19) encoding Protein ID - P38225 (SEQ ID No: 20)) in *Saccharomyces cerevisiae*; fatty acid transporter gene (GeneID: 2911572 (SEQ ID No: 21) encoding Protein ID - Q6C5Q8 (SEQ ID No: 22)) in *Yarrowia Lipolytica*; fatty acid transporter gene (GeneID: 8197297 (SEQ ID No: 23) encoding Protein ID - C4QXD6 (SEQ ID No: 24)) in *Pichia pastoris*; and fatty acid transporter gene (GeneID: 8302036 (SEQ ID No: 25) encoding Protein ID - C5MBJ9 (SEQ ID No: 26)) in *Candida tropicalis*.

2.2.0 A genetically modified yeast or fungal species/strain with increased fatty acid anabolism

2.2.1 A genetically modified yeast or fungal strain with increased production of cytosolic Acetyl CoA.

A yeast or fungal strain of the invention is genetically modified by the transformation with and expression of a gene encoding Pyruvate-Formate lyase (pfl), which is an enzyme which converts pyruvic acid to acetyl-CoA and formic acid in the cytosol. PFL function in yeast requires expression of both the structural gene encoding the PFL homodimer (pflB) and its activating enzyme (pflA), and single electron donor as co-factor. Inactive PFL is converted into its active form under anaerobiosis by the stabilization of a glycy radical in its active site, a process which is mediated by PflA. *S. cerevisiae* contains a cytosolic, single-electron donor capable of activating PFL. In one example the Pfl A and PflA gene from *E. coli* are transformed

into the yeast or fungal strain. Since the availability of acetyl-CoA may function as one of the important constraints in the biosynthesis process of not only fatty alcohols but lipids in general, this genetic modification serves to boost the production of fatty acids by the fermenting organism.

5 Yeasts and fungi are eukaryotic organisms, in which many cellular processes are compartmentalization, such that the bulk of lipid-biosynthesis is located in cytosol, while catabolism is located in mitochondria and peroxisomes. Glycolysis, which leads to production of pyruvate, is localised in the cytosol of yeast and fungi, while the conversion of pyruvate to acetyl CoA takes place in
10 mitochondria catalysed by pyruvate dehydrogenase complex. Thus, the concentration of acetyl-CoA is relatively lower in cytosol than in mitochondria. Pyruvate-Formate lyase is a "Bypass" for mitochondrial oxidation of pyruvic acid, converting pyruvate (formed by Glycolysis in cytosol) into formate and acetyl CoA. Since this enzyme is localised in the cytosol, it can be used to
15 increase the concentration of acetyl CoA in the cytosol and more acetyl CoA is available for conversion into Malonyl CoA by ACC1 as described below under point 2.2.4.

The aim of the genetic modifications set out above under points 2.1.1; 2.1.2; 2.1.3; and 2.2.1, and below under point 2.2.4., is to maximise the number of
20 carbon moles ingested by the fermenting organism that are forced to enter the lipid biosynthesis pathway.

2.2.2 A genetically modified yeast or fungal species/strain with improved redox balance by deletion of Formate dehydrogenase (FDH) gene

25

The yeast or fungal strain of the invention that has been genetically modified to express pyruvate-Formate lyase (PFI A&B) (see point 2.2.1 above), will produce formate or formic acid in the cytosol. The yeast and fungal genome comprises a gene encoding formate dehydrogenase, which degrades
30 formate to carbon dioxide and water, with production of an NADH molecule. However, while growing on glycerol, there already is one NADH which is

produced in pre-glycolytic oxidation of glycerol-Phosphate to Phosphoglyceraldehyde. The fatty acid biosynthesis reaction consumes two NADPH, while growth on glycerol produces two NADH. Coupled with activity of Pyruvate Formate Lyase and Formate Dehydrogenase, there will be four
5 NADH produced per molecule of glycerol. To avoid this redox imbalance, the FDH gene is deleted, thus maintaining the redox balance of the metabolic system. In one example the FDH gene to be deleted is the FDH gene (GeneID: 854570 (SEQ ID No: 44) encoding Protein ID - Q08911 (SEQ ID No: 45)) in *Saccharomyces cerevisiae*; the FDH gene (GeneID: 8300341
10 (SEQ ID No: 46) encoding Protein ID - C5M8W6 (SEQ ID No: 47)) in *Candida tropicalis*, and the FDH gene (GeneID: 2907923 (SEQ ID No: 48) encoding Protein ID - Q6BZU8 (SEQ ID No: 49)) in *Yarrowia lipolytica* .

2.2.3 A genetically modified yeast or fungal species/strain with improved
15 hydrogen production coupled to redox balance

The yeast or fungal strain of the invention that has been genetically modified to express pyruvate-Formate lyase (PFI A&B) (see point 2.2.1 above), and from which the FDH gene has been deleted (see point 2.2.2 above), will produce formate or formic acid in the cytosol. Although the redox imbalance
20 due to FDH activity is avoided by deletion of the FDH gene (see point 2.2.2), there remains the problem of an extra NADH, produced by PFI Activity. Furthermore, in higher concentrations, formic acid is toxic to the fermenting yeast cell. Thus, heterogenous expression of a bacterial formate-hydrogen lyase gene in the genetically modified yeast or fungal strain described under
25 of 2.2.1 and 2.2.2 above (for example by transformation with the formate-hydrogen lyase A gene from E.coli (GeneID: 8486957 (SEQ ID No: 50) encoding Protein ID - C8UET5 (SEQ ID No: 51)) will secure coupled hydrogen production and redox equilibrium in the cell. Formate-Hydrogen lyase degrades formate molecule into hydrogen gas and carbon dioxide
30 under anaerobic conditions. In the process of doing that, the enzyme utilizes one NADH as reducing equivalent. Thus, by this enzyme both the problems

of Redox-imbalance and formate toxicity are solved. Furthermore, since the activity of this enzyme is optimum under anaerobic conditions, just like cytosolic pfl and ACC1, the activity of this enzyme will be optimal under the dual-phase fermentation method for the production and secretion of fatty acids according to the present invention.

2.2.4 A genetically modified yeast strain with increased production of malonyl CoA.

Acetyl-CoA functions as a metabolic “junction”, interconnecting several metabolic pathways, both anabolic and catabolic. Acetyl CoA Carboxylase (ACC) catalyses the reaction in which the Acetyl CoA is carboxylated to form a 3-carbon compound “Malonyl CoA”, which is then committed to enter the metabolic pathway of lipid biosynthesis. Thus, a rise in the cytosolic concentration of Malonyl CoA in fermenting yeast will result in a higher flux through lipid-biosynthesis and higher production of fatty acids. The concentration of malonyl CoA in the modified yeast strain may be increased by over-expressing the ACC1 gene encoding an acetyl CoA Carboxylase which converts acetyl CoA into Malonyl CoA. In one example enhanced expression/synthesis of acetyl CoA Carboxylase can be obtained by manipulating the expression levels of the native ACC encoding gene in its host cell, for example by substituting the native ACC gene promoter with an alternative promoter that directs higher expression levels of the cognate ACC gene. Over-expression of the ACC1 gene in yeast can, for example, be achieved by replacing the endogenous promoter of the native ACC1 gene with the TEF1 promoter from *Saccharomyces cerevisiae* [SEQ ID NO: 113]. Suitable AAC1 genes to over-express include the ACC1 gene (GeneID: 855750 (SEQ ID No: 27) encoding Protein ID - Q00955 (SEQ ID No: 28)) in *Saccharomyces cerevisiae*; the ACC gene (GeneID: 2543344 (SEQ ID No: 29) encoding Protein ID - P78820 (SEQ ID No: 30)) in *Schizosaccharomyces pombe*; the ACC gene (GeneID: 8196923 (SEQ ID No: 31) encoding Protein ID - C4QXW1 (SEQ ID No: 32)) in *Pichia pastoris*; the ACC gene (GeneID:

8301221 (SEQ ID No: 33) encoding Protein ID - C5M4L7 (SEQ ID No: 34)) in *Candida tropicalis*; the ACC gene (GeneID: 2909424 (SEQ ID No: 35) encoding Protein ID - Q6CC91 (SEQ ID No: 36)) in *Yarrowia lipolytica*; and the ACC gene (GeneID: 2871016 (SEQ ID No: 37) encoding Protein ID - C8V2U7 (SEQ ID No: 38)) in *Aspergillus nidulans*.

2.3.0 A genetically modified yeast or fungal species/strain with increased production of short chain fatty acids, e.g. lauric acid.

The fatty acids secreted by the yeast or fungal species/strain of the invention is preferably shorter than 16 carbons in length, preferably 14 or 12 carbons in length. Fatty acid chain length is determined by the cytosolic enzyme, thioesterase (Acyl CoA-ACP Thioesterase), which cleaves the bond between growing fatty acid chain on the Fatty acid Synthase Complex (FAS) and releasing the fatty acid in the cytosol. Typically in yeast, the native Acyl CoA-ACP thioesterase cleaves the thioester bond between Fatty acyl-CoA and Acyl-Carrier Protein (ACP) when the fatty acid chain reaches 16 Carbons in length. However, certain other oil-plants such as Palm and Cinnamon have a native thioesterase that can cleave the thioester bond of a growing fatty acid chain when it reaches 12 carbons in length. Transformation of a yeast or fungal species/strain of the invention with a gene encoding Acyl CoA-ACP thioesterase from *Cuphea wrightii*, *Umbrelia californica*, *Cinnamomum camphorum*, Soyabean, will increase the proportion of medium-chain fatty acids, thereby increasing the quality of the fuel-mixture derivable therefrom. In one example the yeast or fungal strain of the invention is genetically modified by transformation with a Acyl CoA-ACP thioesterase gene derived from any on of the following: Soyabean (*Glycine max*) GeneID: 100170693; *Chlamydomonas reinhardtii* (GeneID: 5722109 (SEQ ID No: 52) encoding Protein ID - A8HY17 (SEQ ID No: 53)); *Arabidopsis thaliana* (GeneID: 837372 encoding Protein ID - Q9SJE2 (SEQ ID No: 54)); *Ricinus communis* (GeneID: 8269197 (SEQ ID No: 55) encoding Protein ID - B9RAC3 (SEQ ID No: 56)); *Triticum aestivum* (GeneID: 543005); CtFatA from *Brassica napus*

(Genbank accession number: X73849 (SEQ ID No: 57) encoding Protein ID - Q43745 (SEQ ID No: 58)); CtFatA from *C. tinctorius* (Genbank accession number: M96569 (SEQ ID No: 59) encoding Protein ID - Q42715 (SEQ ID No: 60)); GmFatA1 from *G. mangostana* (Genbank accession number: U92876 (SEQ ID No: 61) encoding Protein ID - O04792 (SEQ ID No: 62)); CwFatB1 from *C. hookeriana* (Genbank accession number: U17076 (SEQ ID No: 63) encoding Protein ID - Q39513 (SEQ ID No: 64)); CwFatB1 from *C. wrightii* (Genbank accession number: U56103 (SEQ ID No: 65) encoding Protein ID - Q39662 (SEQ ID No: 66)); GmFatB1 from *G. Mangostana* (Genbank Accession number: U92878 (SEQ ID No: 67) encoding Protein ID - O04794 (SEQ ID No: 68)).

The expression of the thioesterase in the transformed yeast or fungal strain of the invention further enhances the esterification of the released fatty acids to ethyl esters.

15

2.4.0 A genetically modified yeast or fungal species/strain with increased secretion of fatty acid esters

Yeasts, such as *Saccharomyces cerevisiae* have the native capacity to secrete esterified fatty acids, such esters being preferred because they are chemically similar to "biodiesel". The enzyme Acyl-coenzymeA:ethanol O-acyltransferase converts fatty acids into ethyl esters of fatty acids, and thus overexpression of Acyl-coenzymeA:ethanol O-acyltransferase will enhance the secretion of fatty acid esters. Expression levels of the native Acyl-coenzymeA:ethanol O-acyltransferase gene (e.g. *Saccharomyces cerevisiae* (GeneID: 856010 (SEQ ID No: 71) encoding Protein ID - Q02891 (SEQ ID No: 72)); *Pichia Pastoris* (GeneID: 8196549 (SEQ ID No: 69) encoding Protein ID - C4QX24 (SEQ ID No: 70)) in a yeast strain of the invention can be achieved by replacing the native promoter of these genes with a stronger promoter.

30

2.5.0 A genetically modified yeast or fungal species/strain with an increased pool of free fatty acids

When the synthesis of a fatty acid is terminated and the fatty acid-chain is cleaved from the Fatty acid Synthase complex (FAS) by the activity of Acyl CoA-ACP thioesterase, the chain of fatty-acyl-CoA is released in cytosol. The enzyme Acyl-CoA Thioesterase that converts the fatty acyl-CoA chains into free fatty acids by cleaving off the CoA group is localized in the peroxisomes in yeast. The release of free fatty acids in the cytosol and recycling of Coenzyme A is enhanced in a genetically modified yeast or fungal species of the invention by the heterogenous expression of the cytosolic mammalian Cytosolic Acyl CoA thioesterase (CTE). A genetically modified yeast or fungal strain of the invention is transformed with a CTE gene derived from: *Mus musculus* (GeneID: 26897 (SEQ ID No: 73) encoding Protein ID – O55137 (SEQ ID No: 74)); *Arabidopsis thaliana* (GeneID: 827955 (SEQ ID No: 75) encoding protein ID - Q5FYU1 (SEQ ID No: 76)); or *Rattus norvegicus* (GeneID: 170588 (SEQ ID No: 77) encoding Protein ID – Q6AZ44 (SEQ ID No: 78)).

2.6.0 A genetically modified yeast or fungal species/strain with an decreased ethanol biosynthesis

Carbon flux into ethanol synthesis is reduced in a genetically modified yeast or fungal strain of the invention, by means of the deletion of the native alcohol dehydrogenase genes, ADH1 and/or ADH5. Expression of the native alcohol Dehydrogenase gene in the genetically modified yeast or fungal strain of the invention is reduced by deleting one of the following genes: ADH1 from *Saccharomyces cerevisiae* (GeneID: 854068 (SEQ ID No: 79) encoding protein ID P00330 (SEQ ID No: 80)); ADH1 from *Schizosaccharomyces pombe* (GeneID: 2538902 (SEQ ID No: 81) encoding Protein ID P00332 (SEQ ID No: 82)); ADH1 from *Aspergillus nidulans* (GeneID: 2868277 (SEQ ID No: 83) encoding Protein ID C8VL73 (SEQ ID

No: 84)); ADH5 from *Saccharomyces cerevisiae* (GeneID: 852442 (SEQ ID No: 85) encoding Protein ID – P38113 (SEQ ID No: 86)).

2.7.0 A genetically modified yeast or fungal species/strain capable of growth on pentose and/or hexose sugars as carbon source.

The genetically modified yeast or fungal strain of the invention expresses a heterologous bacterial Glycerol Kinase and/or Xylose Isomerase genes such that the strain is capable of growth on lignocellulose derived substrates (e.g. pentose and/or hexose sugars) and/or glycerol as carbon source for production of biodiesel. These heterologously expressed enzymes serve to funnel the carbon flux towards fatty acid lipid biosynthetic pathway. A genetically modified yeast or fungal strain of the invention is transformed with a gene selected from one or more of: Xylose isomerase from *Clostridium phytofermentas* (GeneID: 5743318 (SEQ ID No: 87) encoding Protein ID - A9KN98 (SEQ ID No: 88)); Xylose Isomerase from *Yersinia pestis* – (GeneID: 1176874 (SEQ ID No: 89) encoding Protein ID - Q8Z9Z1 (SEQ ID No: 90)); and Glycerol Kinase from *Saccharomyces cerevisiae* (GeneID: 856353 (SEQ ID No: 91) encoding Protein ID - P32190 (SEQ ID No: 92)).

3.0 Method for enhanced production and extracellular secretion of fatty acids and their esters by the yeast strains of the invention.

In a first embodiment, the growth of the yeast strain of the invention and extracellular secretion of fatty acids and their esters by the yeast strain, is obtained by a Dual-Phase fermentation method. According to this method, a selected growth medium is first inoculated with cells of the yeast strain of the invention. The inoculated culture is then incubated for a total period to be selected from between at least 1 hour to up to 200 hours, for example at least 3, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 100, 120, 140, 160, or 180 hours. Preferably the total incubation period was about 84 hours, this being the optimum for obtaining the greatest yield of secreted fatty acids,

while longer incubation periods were accompanied by decrease in yield, probably due to auto-digestion of the secreted fatty acids by the fermenting organisms. In the first phase of the incubation period, the growth medium is initially aerated (defined as an oxygen saturated growth medium
5 aerated/oxygenated to 100, 95, 90,85,80,75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 15, 10, 5, 1, 0.1%) for a period sufficient to allow aerobic growth of the yeast strain. Saturated oxygen conditions can be obtained by supplying a flow of oxygen at an uptake rate of 1.20 mmol/g/h and shaking the culture at a rate of about 100-120 rpm. The aerobic growth conditions
10 are maintained for period of time to be selected from at least 1 hour to up to 20 hours, for example at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18 or 20 hours. The aeration of the growth medium is reduced at the end of the selected aerobic growth period, by partial or complete cessation of the oxygen supply. As a consequence, the growth medium that was initially
15 saturated with oxygen gradually becomes anaerobic as the oxygen levels decline, causing the cells of the yeast strain to enter a second anaerobic growth phase. By the end of aerobic growth period, the culture typically reaches an optical density of 4 to 5, providing a sufficient number of cells entering the anaerobic stage of respiration, where the cells start secreting
20 and releasing fatty acids in the medium. The OD tends to vary with different yeast strains depending upon their specific growth rate.

As a further embodiment of the above described Dual-Phase fermentation method, the temperature at which the yeast strain of the invention is
25 incubated is maintained at a constant temperature of between 15 and 45°C, for example 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40 or 45°C, or any temperature value lying in between the above values. Preferably the temperature is maintained at between 25 and 35°C.

30 As a further embodiment of the above described Dual-Phase fermentation method, the pH of the growth medium in which the yeast strain are cultured is

maintained at a constant pH of between 2.5 and 4.5, for example 2.8, 3.0, 3.2, 3.4, 3.6, 3.8, 4.0, 4.2 or 4.5, or any pH value lying in between the above values. Preferably the pH is maintained at between 3.0 and 4.5. The pH can be maintained by HCl/NaOH regulation, where the pH of the culture is periodically checked and the pH then adjusted to 3.5 by adding appropriate amounts of NaOH or HCl. When sodium citrate buffer was used to maintain pH, the yields of fatty acids decline, due to the inhibitory effect of citrate on fatty acid synthesis.

As a further embodiment of the above described Dual-Phase fermentation method, the growth medium in which the yeast strain are cultured is a synthetic medium designed to exert maximum osmotic stress, by providing a high sugar (e.g. 200 g/l dextrose) concentration, combined with a source of nitrogen (e.g. ammonium sulfate) and essential vitamins of the B complex group. In a preferred embodiment, the medium is based on Wickerham synthetic medium (1951, ref), which is modified to exert osmotic stress. Preferably the medium is supplemented with a carbon source such as glycerol, glucose, pentose sugars at a preferred concentration of: 1% to 5% glycerol; 15% to 20% glucose concentration; and 15% to 20% pentose.

As a further embodiment of the above described Dual-Phase fermentation method, the fermentation is carried out in a bioreactor provided with a controllable means for supplying the culture with oxygen, to facilitate both aerobic and anaerobic growth phases. Further the bioreactor is one that is provided with a means for stirring the culture, and preferably allows for the continuous introduction of nutrients. Preferably, the bioreactor is provided with means for separating the extracellular medium from the yeast cells.

4.0.0 Production of alkanes from free fatty acids by fatty acid decarboxylation

Free fatty acids secreted by the genetically modified yeast or fungal species/strains of the invention may be converted to alkanes by a variety of chemical steps as set out below:

5 4.0.1 Barton Decarboxylation

Free fatty acids may be catalytically converted alkanes by decarboxylation by the means of Barton Decarboxylation (<http://www.organic-chemistry.org/namedreactions/barton-decarboxylation.shtm>) OR Hunsdiecker Reaction (<http://www.organic-chemistry.org/namedreactions/hunsdiecker-reaction.shtm>).
10

4.0.2 Kolbe electrolysis

Free fatty acids may be converted to alkanes by electrolytic decarboxylation by means of Kolbe electrolysis. (<http://www.organic-chemistry.org/namedreactions/kolbe-electrolysis.shtm>).
15

4.0.3 Palladium/Platinum (PD/C) catalyst

Free fatty acids may be converted to alkanes by treatment with a PD/C catalyst at 300°C and pressure 12 bars for 4 hours.
20

4.0.4 Enzymatic decarboxylation

Free fatty acids may be converted to alkanes by decarboxylation with immobilized enzymes, which may be prepared from extracts of cells comprising suitable enzymes such as the insects: *Apis mellifera*, *Musa domestica*, *Zootermopsis angusticollis*, *Triatoma infestans* which contain native long-chain fatty acid-decarboxylase for decarboxylation of fatty acids longer than 20 carbon in chain-length. Further suitable enzymes may be obtained from extracts of the algae: *Crocospaera spp*, *Isochrysis*, *Prymnesium spp*, *Ectocarpus spp*, *Laminaria spp*, *Streblonema spp*. The
25
30 genetically modified yeast cells of the invention, when grown on the modified growth medium of the invention are known to secrete fatty acids with chain-

length longer than 20 carbon atoms. The presence of Long-Chain Fatty acids (LCFAs) decreases the quality of the biodiesel by increasing its density and increasing the freezing point of the fuel-mixture, increasing their tendency to freeze in colder weathers. To avoid this and to increase the calorific value of the fuel, the LCFAs are decarboxylated enzymatically by the means of LCFA decarboxylases present in the extracts of various insects like "honey bee" (Apis mellifera), Wood-termite (Cootermopsis angusticollis), Triatoma etc. The Fatty-acid mixture, secreted by the yeast of the invention is treated with enzymes present in the immobilized extracts of these insects along with NADPH solution is treated on in the medium. The proportion of LCFAs in the medium with chain-length of 20, 22, 24 carbon atoms will be decarboxylated to yield the corresponding alkanes with one carbon less than the parent chain-length. Thus, the resultant fuel mixture is of higher calorific value.

Example 1. Optimal growth conditions, with respect to temperature, pH, media composition and dextrose concentration, for fatty acid production by yeast

Growth conditions that maximise fatty acid production in yeast were tested, with a view to identifying conditions that modulate biomass increase and those that enhance fatty acid synthesis and accumulation and/or secretion.

Two strains of the yeast, *Saccharomyces cerevisiae* were tested in parallel:

- 1) National centre for industrial microorganisms (NCIM) strain 3090, (<http://www.ncl-india.org/ncim/cataloguedetails.jsp?mid=29&category=yeast&ncimno=3090>)
- 2) Ordinary baker's yeast strain.

The strains were grown on Wickerham Synthetic Medium (Wickerham L. J. 1951. Taxonomy of yeast. US Department of Agriculture, Technical Bulletin No.1029.) whose composition is set out in Table 1, but modified as indicated with respect to carbon source.

Table 1

Component	Composition (grams/litre)
Yeast extract	6.7 g
Carbon Source	
Dextrose	200 g
Nitrogen Source	
Ammonium sulphate	5 g
Vitamins*	
Biotin	20 µg
Calcium pantothenate	2 µg
Folic acid	2 µg
Inositol	10 mg
Niacin	400 µg
Riboflavin	200 µg
Pyridoxine hydrochloride	400 µg
Thiamine hydrochloride	400 µg
Compounds supplying trace elements	
Boric acid	500 µg
Copper sulphate	40 µg
Potassium iodide	100 µg
Ferric chloride	200 µg
Manganese sulphate	400 µg
Sodium molybdate	200 µg
Zinc sulphate	400 µg
Salts	
Potassium phosphate monobasic	850 mg
Potassium phosphate dibasic	150 mg
Magnesium sulphate	500 mg
Sodium chloride	100 mg
Calcium chloride	100 mg
Wickerham Synthetic Medium (Wickerham et al 1951 supra)	

Fermentation conditions:

- 5 i) Dual-Phase fermentation of growth medium inoculated with either of the two strains was carried out in 1-liter bioreactors for a period of 84 hours. The growth medium was initially saturated with oxygen for the first 10 hours after inoculation. The oxygen supply to the

reactor was then stopped such that the yeast cells gradually entered an anaerobic growth phase, as anaerobic conditions build up in the bioreactor as the oxygen level declined. Un-inoculated fermentations were carried out in parallel as a control for comparison with inoculated cultures.

5

ii) The effect of growth temperature was analysed by adjusting the temperature of the growth medium to a temperature ranging from 15 to 45°C, as indicated in Figures 1a. In all other tested fermentation conditions the growth medium in the bioreactor was maintained at a temperature of 30°C.

10

iii) The effect of glucose concentration was analysed by supplementing the fermentation medium with glucose as the carbon source in place of dextrose in a final concentration of between 5 and 20 g/liter, as indicated in Figure 1b.

15

iv) The effect of pH was analysed by adjusting the pH of the fermentation medium by addition of HCl or NaOH, to obtain a pH of between 2.0 and 5.0, as indicated in Figure 1c.

Analysis of fatty acid production:

20 After 84hr fermentation, the yeast cells from each fermentation were separated from the fermented broth by centrifugation. Fatty acids were extracted from the cells by the method described by Cocito, C and Delphini, C. 1994, in *Food Chemistry*, **50**(3), 297-305. Fatty acids, in the growth medium or extracted from the cells, were saponified using aqueous or
25 alcoholic KOH, and the saponified fatty acids were recovered by centrifugation, and subsequently acid-treated with concentrated HCl to produce free fatty acids. The fatty acids were then separated from the aqueous phase using solvent extraction with diethyl ether or chloroform. The extracted fatty acids were subjected to gas chromatographic analysis to
30 identify the fatty acids composition and their chain-lengths.

Gas Chromatography (GC) conditions:

Methyl Esters of the fatty acid samples were prepared and injected (volume: 1.0 ml) into the GC Instrument: [Chemito GC 8610; equipped a 10% FFAP 3 m column with an O.D: 1/8, Mesh- 80/100; and a FID Detector: FID] using 5 chloroform as solvent. The temperature Program: Initial temperature 1:100°C; hold 1:2 min; rate 1:10°C; temperature 2: 250°C; hold 2:25 min; Injection/Detector temperature: 250°C/250°C; carrier Flow: 30 ml/min; range: x10; attenuation: x1;

10 **Table 2:** Fatty acids content of the sample measured by GC, where the peaks in the chromatogram (Figure 2), can be compared with the values in the table. The value corresponding to the peak signifies the presence of that particular chain length fatty acid (given in last column of the table)

Peak No.	Retention Time	Area [mV.s]	Height [mv]	W05 [min]	Area [%]	Height [%]	Result (MCFA's)
1	6.387	5.4011	0.359	0.253	0.124	0.153	Caproic Acid (C6)
3	9.393	40.9518	2.783	0.240	0.938	1.186	Caprylic Acid (C8)
5	12.207	162.2671	10.256	0.253	3.716	4.373	Capric Acid (C10)
7	14.773	559.4485	36.779	0.233	12.813	15.681	Lauric Acid (C12)
11	17.120	263.1979	17.757	0.240	6.028	7.571	Myristic Acid (C14)
14	19.773	607.1143	32.803	0.307	13.904	13.986	Palmitic Acid (C16)

15 **Conclusions:** The conditions that are most conducive for production and extra-cellular secretion of Medium Chain Fatty Acids (MCFA) by the two

strains of *Saccharomyces cerevisiae* when grown in Wickerham Synthetic Medium are as follows:

Table 3: Optimal growth conditions for MCFA production by yeast

Parameters	Value
Temperature	25°C
pH	3.5
Glucose Concentration	18 g/liter
Fermentation conditions	Initially aerobic, later anaerobic

5

MCFAs were secreted extra-cellularly into growth medium, where both the MCFAs and their ethyl esters are candidates for use as Biodiesel.

10 While not by theory, it is believed that fatty acid synthesis in micro-organisms (including yeast) adapts the membrane fatty acid profile (in particular its phospholipid content) in order to maintain its fluidity in response to changing growth temperature. Higher temperatures stimulate synthesis of longer chain FAs with a higher boiling point, while lower temperatures stimulate synthesis of lower chain length FAs with lower density and boiling point.

15

Increasing the sugar concentration in the fermentation medium appears to activate the lipid biosynthesis machinery, where it is believed that excess Acetyl CoA produced by glycolysis, is diverted to malonyl CoA production which in turn leads to FA biosynthesis. A high glucose concentration also exerts osmotic pressure on the plasma membrane of the yeast cells, to which the membrane adjust by becoming more resilient. This is achieved by increasing the content of MCFA's and unsaturated FA's [UFAs] in membrane. whereby the kinks in the unsaturated chain cause the molecules to interlock, thereby increasing their resilience.

20

25 The production and secretion of FAs is greatest around pH 3.5, and their secretion cause the pH to decrease due to their acidity. However, with time,

pH starts increasing again, which is thought to be due esterification of the free fatty acids that are produced. Thus both MCFAs and their ethyl esters are seen to accumulate in the growth medium.

5 **Example 2. Deletion of FAA2 gene from *Saccharomyces cerevisiae* enhances the production of fatty acids**

2.0.0 Methods

The FAA2 gene was deleted *Saccharomyces cerevisiae* strain CEN-PK2
10 (MATa/MATa; ura3-52/ura3-52; trp1-289/trp1-289; leu2-3,112/leu2-3,112; his3 D1/his3 D1; MAL2-8C/MAL2-8C; SUC2/SUC2) obtained from strain collection of CSM, and replaced with the 1.1 kbp URA3 marker gene (derived from *Kluyveromyces marxianus*) conferring the capacity to synthesize uracil. URA3 encodes orotidine 5-phosphate decarboxylase (ODCase), an enzyme
15 involved in the synthesis of pyrimidine ribonucleotides.

Primer set one was designed to delete FAA2 gene along with 150 base-pairs flanking the FAA2 gene upstream, and 60 base-pairs flanking the FAA2 downstream in the parent CEN-PK2 strain (henceforth called WT-strain). Primer set 3, which binds near the centre of the FAA2 gene sequence, was
20 used to amplify a ~1300 bp fragment of the FAA2 gene, in order to detect the FAA2 gene in genomic DNA from the WT-strain. The primers were designed using Primer-Design Tool available on www.yeastgenome.org.

Table 4: List of primers used for the purpose of gene deletion

<p><i>Primer set</i> 1 (for FAA2 deletion)</p>	<p><i>Upstream flank</i> – CAGCTATGACCATGATTACGGGAGTCAAAAACGAGGGAACTCAATATTAATTGCGGAGTTGAGCGAT GATGGGTTGGAACATATAAAGCATCGGAAACGCATGGCTAAGGGAAGTGAAGAATGCAGGTTC CAAAAACGGATAAGAACAACAACTTGTTCGAAAT - FAA2</p> <p><i>Downstream Flank</i> – FAA2 - GTACTTATGACGATTTGGAACACATTCAAACCTAGAAAAAACTTTGATGTAGGATATCCCTTTTTGAT CGGGTAATAACTG</p>
<p><i>Primer set</i> 2 For URA3 amplification)</p>	<p>URA3 Upstream flank - fw: TGACCATGATTACGAATTAG; Rv: CTTCAAACCGCTAACAATAC</p>
<p><i>Primer Set</i> 3 (For FAA2 amplification)</p>	<p>FAA2 Fw: ATGGCCGCTCCAGATTATGC ; FAA2 Rv: TGCAAAAATCAGAATGGGGG</p>

Primer set 1: upstream: SEQ ID NO: 93; downstream: SEQ ID NO: 94,

Primer set 2: URA3 upstream flank fw: SEQ ID NO: 95; Rv: SEQ ID NO: 96,

Primer set 3: FAA2 Fw: SEQ ID NO: 97; FAA2 Rv: SEQ ID NO: 98.

- 5 WT-strain genomic DNA was extracted and subjected to Fusion PCR where the URA 3 gene was inserted in place of the deleted FAA2 gene. The yeast cells were transformed with URA3 according to protocol described by Gietz (Gietz, Jean et al. 1992). The transformed colonies were grown on SD-URA minimal medium plates. Colony PCR was conducted using Primer set 2, 10 which clearly demonstrated the presence of the inserted URA3 gene and the loss of FAA2 gene in the mutant colonies. Stable mutant colonies were propagated on minimal media plates.

15 The transformed yeast strains were grown in YPD medium for 2 days to an O.D of 2 and subsequently, the growth experiments were conducted in Modified Wickerham's synthetic media, as given in Table 5, for 6 days.

Table 5

Component	Composition (grams/litre)
Carbon Source	
Dextrose (or alternative carbon source*)	180 g (or as defined)
Nitrogen Source	
Ammonium sulphate	5g
Vitamins	
Biotin	20 µg
Folic acid	2 µg
Inositol	10 mg
Niacin	400 µg
Riboflavin	200 µg
Pyridoxine hydrochloride	400 µg
Thiamine hydrochloride	400 µg
Compounds supplying trace elements	
Boric acid	500 µg
Copper sulphate	40 µg
Potassium iodide	100 µg
Ferric chloride	200 µg
Manganese sulphate	400 µg
Sodium molybdate	200 µg
Zinc sulphate	400 µg
Salts	
Potassium phosphate monobasic	850 mg
Potassium phosphate dibasic	150 mg
Magnesium sulphate	500 mg
Sodium chloride	100 mg
Calcium chloride	100 mg
Modified Wickerham Synthetic Medium	

*alternative carbon sources: glycerol, xylose, digested cellulose and hemicelluloses, starch, mannitol and other sugar-alcohols, xylan.

5 The fermentation conditions were multiphase comprising an initial 48 hours of aerobic growth, followed by 4 days of anaerobic conditions to facilitate the release of fatty acids into the medium. The flasks were incubated in a water-bath at 30°C and shaken at a speed of 80 rpm for efficient mixing. After 6 days fermentation, the OD of the culture was measured and the cells were then removed from the liquid phase by centrifugation at 5000 rpm for 6

minutes. The intra-cellular and extra-cellular fatty acids in the cells and the supernatant were extracted and quantified, according to Cocito and Delfini, 1994 *supra*. The only deviation from the protocol was that the extracellular fatty acids were extracted in the solvent diethyl ether instead of chloroform.

5

GC conditions for analysis of fatty acids: employed a DBI capillary column, (30 m long and 0.25 mm i.d; film thickness 0.25 μ m), with a temperature gradient of 40°C to 200°C at 6°C/min; 200°C for 15 minutes; 200°C to 260°C at 6°C/min; 260°C to 290°C at 2°C/min; an injector temperature of 280°C; detector temperature of 300°C; a split rate of 1:20; using the carrier gas helium; and linear flow-rate of 1.5ml/min; pressure 15.7 psig; and an injection volume of 1-2 μ l.

10

Calculation of fatty acid yield: Area under the peak of particular component determines its percentage in the given mixture. The total percentage of fatty acids per 50ml of extracted lipids is based on the sum of the percentages of largest components (those above 4% of yield). Subsequently, the proportion of those fatty acids per litre is calculated by means of simple cross-multiplication.

15

20

2.1.0 Production of a FAA2 deletion strain (FAA2 Δ ura3) in *Saccharomyces cerevisiae*, WT-strain.

The presence of FAA2 gene in the genome of WT strain was demonstrated by PCR employing primer set 3, where the amplified FAA2 gene fragment was separated and detected by gel electrophoresis (figure 5). Following transformation of the WT-strain, to delete the FAA2 gene, the transformed cells were grown on selective media plate (SD-URA). The growth of 16 colonies on SD-URA plates denoted successful transformation to yield Δ ura3 yeast strains. Genomic DNA, extracted from the deletion mutants, was

25

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subjected to PCR amplification using FAA2 primers and URA3 primers to confirm the Δ ura3 deletion event, Figure 6a,b.

The FAA2 gene was not detected in transformed colonies that were amplified with primer set 3 confirming the deletion of the FAA2 gene from the genome
5 of the mutant yeast (wells 3-9, 11-17 in fig 6a,b). The URA3 gene was clearly detected in several of the mutants colonies (wells 25, 26, 8, 30-33 in Fig 6a,b) amplified with primer set 2 thereby demonstrating the Δ ura3 deletion event. The FAA2 deletion strain (FAA2 Δ ura3) in *Saccharomyces cerevisiae* (AGPH-01) is deposited under the strain name CBS126804 with
10 Centraalbureau voor Schimmelcultures, P.O Box 85167, 3508 AD Utrecht, NL on 26.04.2010 in conformity with rule 9.1 and 11.4(g) of the Budapest Treaty.

**2.2.0 Fatty acid synthesis and secretion profile of FAA2 deletion stain
15 (FAA2 Δ ura3) versus *Saccharomyces cerevisiae* WT-strain.**

The FAA2 Δ -stain and WT-strain were grown on Modified Wickerham's synthetic media with the specified carbon source, and fatty acids were extracted from the growth medium and cells respectively as set out under
(2.0.0)

20 2.2.1. Extracellular fatty acid profile of WT-strain grown on 20% glucose

The Optical Density of the WT strain culture at the time of harvest was 11. The yield of fatty acids at the end of organic extraction of the culture medium was 5 ml per 50 ml of sampled culture medium. A crude sample of extracellularly secreted fatty acids, extracted from the culture medium, was
25 separated by Gas-Chromatography and the components identified using Mass-Spectrometry (GC-MS). The GC-MS graph in figure 7 shows various peaks having retention times (RT) corresponding to components (mostly fatty acids) of the test-sample. All peaks corresponding to more than 4% of the secreted lipids are listed in Table 6.

30

Table 6: Major extracellular fatty acids secreted by WT strain grown on 20% glucose.

Retention Time	Name of the component	Percentage of the component
----------------	-----------------------	-----------------------------

28.79	Palmitic acid (C:16)	4%
30.74	Stearic acid (C:18)	11%
32.04	Tetratetracontane (C:44)	30%
36.08	Tetratetracontane (C:44)	22%

The yield of pure fatty acids secreted by the WT-stain yeast was approximately 67 ml per liter of culture.

5 **2.2.2.** Intracellular fatty acid profile of WT strain grown on 20% glucose

The Optical Density of the WT strain culture at the time of harvest was 11. The yield of intracellular fatty acids extracted from cells from a 50 ml of sample was 0.5ml. A sample of the extracted intracellular fatty acids was analysed by GC-MS. The GC-MS graph in figure 8 shows various peaks
10 having retention times (RT) corresponding to components (mostly fatty acids) of the test-sample. All peaks corresponding to more than 4% of the secreted lipids are listed in Table 7.

Table 7: Major intracellular fatty acids in WT strain grown on 20% glucose

Retention Time	Name of the component	Percentage of the component
28.84	Palmitic acid (C:16)	5%
30.81	Stearic Acid (C:18)	10%
32.48	Oleic acid (C:18,9)	22%

15

2.2.3. Extracellular fatty acid profile of FAA2 Δ ura3 strain grown on 20% glucose

The Optical Density of the culture at the time of harvest was 7. The yield of saponifiable fatty acids at the end of organic extraction of the culture medium
20 was 10 ml per 50 ml of sampled culture medium. A sample of the extracted extracellular fatty acids was analysed by GC-MS The GC-MS graph in figure 9 shows various peaks having retention times (RT) corresponding to components (mostly fatty acids) of the test-sample. All peaks corresponding
25 to more than 4% of the secreted lipids are listed in Table 8.

Table 8: Major extracellular fatty acids secreted by FAA2 Δ strain grown on 20% glucose

Retention time	Name of the component	Percentage of the component
27.34	Pentadecanoic acid (C:15)	5%
28.80	Palmitic acid (C:16)	13%
29.42	Cycloeicosane (C:20)	9.5%
30.76	Stearic acid (C:18)	42%
31.30	?	7.6%

5 The yield of pure fatty acids secreted by the FAA2 Δ strain yeast was approximately 134 ml per liter of culture. Very Long Chain Fatty Acids (VLCFA) are not secreted by the FAA2 Δ strain in contrast to the WT strain of *S. cerevisiae*.

10 **2.2.4.** Intracellular fatty acid profile of FAA2 Δ ura3 strain grown on 20% glucose

The Optical Density of the FAA2 Δ strain culture at the time of harvest was 7.

The yield of intracellular fatty acids extracted from cells from a 50 ml of sample was 0.4ml. A sample of the extracted intracellular fatty acids was

15 analysed by GC-MS The GC-MS graph in figure 10 shows various peaks having retention times (RT) corresponding to components (mostly fatty acids) of the test-sample. All peaks corresponding to more than 4% of the secreted lipids are listed in Table 9.

20 **Table 9:** Major intracellular fatty acids secreted by FAA2 Δ strain grown on 20% glucose.

Retention time	Name of the component	Percentage of the component
28.81	Palmitic acid (C:16)	20%
30.75	Stearic acid (C:18)	23%
32.36	Oleic acid (C:18, 9)	31%

The relative amount of intracellular C18 fatty acids are elevated in the FAA2 Δ strain grown on glucose when compared to the WT strain.

2.2.5. Extracellular fatty acid profile of WT strain grown on 5% glycerol

To assess alternative carbon sources in the growth medium, glucose was replaced by 5% glycerol, while keeping the remaining components of the growth medium constant. The Optical Density of the WT strain culture at the time of harvest was 7. The yield of fatty acids at the end of organic extraction of the culture medium was 4 ml per 50 ml of sampled culture medium. A sample of the extracted extracellular fatty acids was analysed by GC-MS. The GC-MS graph in figure 11 shows various peaks having retention times (RT) corresponding to components (mostly fatty acids) of the test-sample. All peaks corresponding to more than 4% of the secreted lipids are listed in Table 10.

Table 10: Major extracellular fatty acids secreted by WT strain grown on 5% glycerol.

Retention time	Name of the component	Percentage of composition
32.42	Oleic acid (based on GC and RT) (C:18, 9)	74%

The yield of pure fatty acids secreted by the WT strain was approximately 60 ml per liter of culture.

2.2.6. Extracellular fatty acid profile of FAA2 Δ ura3 strain grown on 5% glycerol

The Optical Density of the WT strain culture at the time of harvest was 4. The yield of fatty acids at the end of organic extraction of the culture medium was 2.5 ml per 50 ml ml of sampled culture medium. A sample of the extracted extracellular fatty acids was analysed by GC-MS. The GC-MS graph in figure 12 shows various peaks having retention times (RT) corresponding to components (mostly fatty acids) of the test-sample. All peaks corresponding to more than 4% of the secreted lipids are listed in Table 11.

Table 11: Major extracellular fatty acids secreted by FAA2 Δ strain grown on 5% glycerol.

Retention time	Name of the component	Percentage of the component
26.68	Myristic acid (C:14)	4.1%
28.83	Palmitic acid (C:16)	18.0%
30.78	Stearic acid (C:18)	30.5%
32.93	Octadecamethyl cyclonosiloxane (C:18)	16.6%

The yield of pure fatty acids (including siloxane) secreted by the FAA2 Δ strain was approximately 32 ml per liter of culture (Table 12).

Table 12: Yields of secreted fatty acids by WT-strain and FAA2 Δ strain

Yeast strain	Fatty acid Yield	Carbon-source and Concentration
WT - strain	67 ml / litre	20% glucose
WT- strain	60 ml / litre	5% glycerol
FAA2 Δ strain	134 ml/ litre	20% glucose
FAA2 Δ strain	32 ml / litre	5% glycerol

2.2.7 Summary of the fatty acid synthesis and secretion profile of FAA2 deletion stain (FAA2 Δ ura3) versus *Saccharomyces cerevisiae* WT.

The FAA2 Δ strain provides a 7%-8% increase in secreted pure fatty acids over the WT-strain, when grown on glucose as carbon source. When grown on glycerol, the yield of fatty acids decreases in both the FAA2 Δ - and WT-stains, associated with a slower growth rate on this carbon source. The proportion of pure MCFAs secreted into the medium by the FAA2 Δ strain was also increased by 16% over the WT-strain, if the VLCFA (tetratetracontane) produced by the WT-strain is excluded. The major MCFAs secreted by the FAA2 Δ strain were palmitic acid (C:16:0) and stearic acid (C18:0) and oleic acid (C:18:1). The fatty acid-derivative of siloxane should be taken as the signal for the respective fatty acid in the original sample, due to a reaction between fatty acid esters in the sample with the stationary phase of the chromatography column.

In contrast to the WT strain, the mutant does not produce any trace of very long chain fatty acids (VLCFAs). VLCFAs are solid fats and essentially

unsuitable as biodiesel. The fatty acid elongase system (ELO1, ELO2, ELO3) serves to elongate fatty acids of chain length 16 (MCFA) upward to 20-26 in *Saccharomyces cerevisiae*. The high proportion of VLCFAs produced by the WT-strain suggests that the activity of the elongase system is elevated in presence of FAA2 gene. Deletion of FAA2 gene, surprisingly, stalls this loss of carbon into VLCFAs via the fatty acid elongase system. Rather, the FAA2 deletion causes carbon flux to be channelled away from VLCFAs towards secretion of MCFAs, indicating a key role in determination of chain-length of fatty acid in yeast. The maximum yield of fatty acids, directly suitable for biodiesel synthesis obtained from the FAA2 Δ strain reached as high as 18-20% from glucose. The yield of fatty acids per mole of glycerol was comparable, but slightly higher than for glucose. The growth conditions associated with anaerobic respiration lead to fatty acid secretion.

In conclusion, deletion of the FAA2 gene in yeast serves to inhibit MCFA catabolism and channels carbon flux into MCFAs, and enhances secretion of the fatty acids of this medium chain length (predominantly palmitic (C:16:0), stearic (C:18:0) and oleic (C:18:1) acids) providing a modified yeast strain of the invention adapted for biodiesel production.

20 **Example 3. Use of *Candida tropicalis* for the production of fatty acids suitable for biodiesel**

Candida tropicalis (DTU stain collection) was grown on Modified Wickerham's synthetic media with the specified carbon source, and fatty acids were extracted from the growth medium and cells respectively as set out under (2.0.0).

25 **3.0.0.** When *C. tropicalis* was grown on synthetic medium supplemented with glucose, and the secreted fatty acids extracted and analysed by GC-MS, a total of 21 peaks were detected (figure 13). The most prominent peak, corresponding to 35% of the total extracted fatty acids, had a retention time of 28.84 minutes, identified as palmitic acid. The yield of saponifiable fatty

30

acids after organic extraction was 7.5 ml per 50 of medium. The yield of palmitic acid was 50ml/litre of the medium.

5 **3.0.1.** When *C. tropicalis* was grown on synthetic medium (Table 5) supplemented with 5% glycerol, and the secreted fatty acids extracted and analysed by GC-MS, a total of 19 peaks were detected (figure 14). The most prominent peak, corresponding to 85.4% of the total extracted fatty acids, had a retention time (RT) of 28.92 minutes. Although the RT corresponded to the pesticide 2-Butoxyethyl 2-(2,2 dichlorovinyl)- 3, 3-dimethylcyclopropane carboxylate, the peak was attributed to palmitic acid, on the basis of their
10 respective molecular mass. The yield of saponifiable fatty acids after organic extraction was 7 ml per 50 of medium. The yield of palmitic acid was 120 ml/litre of the medium.

3.0.2 Summary of the fatty acid synthesis and secretion profile of *Candida tropicalis*.
15

C. tropicalis grown on medium supplemented with either glucose or glycerol secretes the MCFA, palmitic acid, during the anaerobic fermentation phase. The yield of secreted palmitic acid is high, corresponding to 120ml/ litre of growth medium. Accordingly, *C. tropicalis* according to the present invention,
20 is a yeast stain that is particularly suited for biodiesel production.

Example 4. Overexpression of Acetyl CoA Carboxylase to increase the malonyl CoA concentration

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4.0.1 Over-expression of the ACC1 gene in yeast is achieved by replacing the endogenous promoter of the native ACC1 gene with the TEF1 promoter from *Saccharomyces cerevisiae* [SEQ ID NO: 113], in order to obtain ACC1 over-expression in a genetically modified yeast. A system for promoter
30 replacement was based on a bipartite DNA molecule in which each of the two DNA fragments carries a target sequence, the sequence to be inserted, and a selectable marker gene, which is non-functional but homologous to some

part of the same marker in the second fragment (Figure 15). The first fragment contains the upstream sequence of ACC1, direct repeat and the upstream 2/3 Kluyveromyces lactis (KI) URA3. The upstream which corresponded to the sequence in front of ACC1 promoter was amplified by primers ACC1 (SWA 3 and SWA4) using genomic DNA of wild type *S. cerevisiae* as a template. Direct repeat with the upstream 2/3 KI URA3 was amplified from pWJ1042 as template with primers SWA5 and 6 (Table 13). Two PCR products were then fused together by primers SWA3 and 6. To obtain the second fragment, three PCR products were generated. The downstream 2/3 KI URA3, which was homologous to 1/3 of KI URA3 in the first fragment, was amplified by using primer SWA7 and 8 (Table 13) from pWJ1042. The TEF1 promoter sequence and the downstream fragment that was homologous to the front part of ACC1 were amplified from yeast genomic DNA by using primers SWA9/10 and SWA11/12 (Table 13), respectively. The three PCR products were then fused by another PCR reaction using primers SWA7 and 12. The above-described two fragments were then be transformed into *S. cerevisiae* and integrated into the yeast chromosome by homologous recombination. The system was constructed so that the URA3 marker was recyclable. To delete the URA3 marker by recombination, transformants were plated on a medium containing 5-fluoroorotic acid (5-FOA). Since Ura3 metabolizes 5-FOA into a toxic compound, yeast that maintain URA3 are killed, whereas yeast lacking URA3 are resistant to 5-FOA and survive. The strain thus created was called a SC-ACC1 strain.

25

Table 13

DNA Fragment	Primer name	Sequence
Upstream of ACC1	SWA3	CACAATTGTTATCGGTTCTAC
	SWA4	GCAGGGATGCGGCCGCTGACCTTGCTCTGAATCTGAATT CC
Direct repeat 1	SWA5	GTCAGCGGCCGCATCCCTGCTTCGGCTTCATGGCAA

and KI URA3		
	SWA6	GAGCAATGAACCCAATAACGAAATC
Direct repeat 2 and KI URA3	SWA7	CTTGACGTTTCGTTGACTGATGAGC
	SWA8	CACGGCGCGCCTAGCAGCGGTAACGCCAGGGTTTTTC
TEF1 promoter	SWA9	CGCTGCTAGGCGCGCCGTGCACACACCATAGCTTCAAAA TGTT
	SWA10	AATAAGCTTTCTTCGCTCATTGTAATTAAACTTAGATT AGA
Downstream of ACC1	SWA11	ATGAGCGAAGAAAGCTTATTCGAGTCTTCTCCACAGAAG ATGGA
	SWA12	TCTCGGAGGCGTGACCCAG

Primer SWA3 – SEQ ID NO: 99; Primer SWA4 – SEQ ID NO: 100; Primer SWA5 – SEQ ID NO: 101; Primer SWA6 – SEQ ID NO: 102; Primer SWA7 – SEQ ID NO: 103; Primer SWA8 – SEQ ID NO: 104; Primer SWA9 – SEQ ID NO: 105; Primer SWA10 – SEQ ID NO: 106; Primer SWA11 – SEQ ID NO: 107; Primer SWA12 – SEQ ID NO: 108.

4.0.2. The higher metabolic flux obtained by overexpression of ACC1 was coupled with inhibition of fatty acid catabolism in form of FAA2 deletion for net increase in production of fatty acids. The SC-ACC1 strain was used as template strand for introducing an FAA2 deletion by replacing it with URA3 gene as described in Example 2. The engineering of the double mutant strain, referred to as SC-FAA2-ACC1, is demonstrated by PCR analysis of gDNA extracted from the mutant strains (Figure 16).

15 **Example 5. Expression of Pyruvate Formate Lyase Pfl (A&B) for increased pyruvate flux.**

The concentration of acetyl CoA in the modified yeast strain is increased by the heterologous cytoplasmic expression of a bacterial gene encoding a Pyruvate formate lyase designated pfl A or pflB enzyme, which converts pyruvate (product of EMP pathway) into formate and acetyl CoA.

The Pyruvate Formate lyase gene (Pyruvate Formate Lyase A (Pfl A) (GeneID: 4491405 (SEQ ID No: 39) encoding Protein ID - A1A9E2 (SEQ ID

- No: 41)) or the Pyruvate formate lyase B (PflB) (GeneID: 4494334 (SEQ ID No: 42) encoding Protein ID - A1A9I0 (SEQ ID No: 43)) from *E. coli* K12) is first cloned on a vector (plasmid). The pflB, pflA, genes are amplified (Figure 17) using their respective 5' and 3' forward and reverse primers (Table 14).
- 5 The genes are cloned by the "biobrick" assembly strategy of prefix and suffix insertions using the restriction enzymes EcoRI, XbaI, SpeI, and PstI. The final constructs consist of pGal1-gene-Adh1_Terminator and are transferred into yeast Tet-off based shuttle vector PCM182 and PCM183 (Figure 18). The assembly is then ready to be transferred to any yeast system for fatty-
- 10 acid production.

Table 14

Primers	Forward	Reverse
PflA	CCTTGAATTCGCGGCCGCATCTAGAATG TCAGTTATTGGTCGCAT	AAGGACTAGTTTAGAACATTACCTTATGAC CGTACTGCTCAAGAATGCC
PflB	CCTTGAATTCGCGGCCGCATCTAGAATG TCCGAGCTTAATGAAAAG	AAGGACTAGTTTACATAGATTGAGTGAAGG TACGAGTAATAACGTCCT GCTG

Primer PflA forward - SEQ ID NO: 109; reverse - SEQ ID NO: 110.

- 15 Primer PflB forward - SEQ ID NO: 111; reverse - SEQ ID NO: 112.

Four *Saccharomyces cerevisiae* strains (wild type and 3 engineered strains) were transformed with both the plasmids PCM182 (PflA) and PCM183 (PflB) and the transformed strains comprise both of the inserted PflA gene and PflB gene [designated PFL system] were selected by growing them on SC-

- 20 TRP agar plates. The four mutant strains created were designated as follows:

S.cerevisiae – PFL system

S.cerevisiae – Δ FAA2 + PFL system

S.cerevisiae – TEF1 Δ ACC1 + PFL system

S.cerevisiae – Δ FAA2 + TEF1 Δ ACC1 + PFL system.

Example 6. Production of fatty acids suitable for biodiesel from different carbon sources employing yeast strains of the invention

6.0.1 Nomenclature of the parent and mutant yeast strains of the invention

5

Strain No.	<i>Saccharomyces cerevisiae</i> strain	Genotype
1	Parent strain - CENPK2	WT
2	FAA2 deletion of WT	Δ FAA2
3	TEF1/ACC1 promoter switch of WT	TEF1 Δ ACC1
4	PFL system (PflA&B genes) inserted into WT	PFL
5	Δ FAA2/ PFL double mutant of WT	Δ FAA2/ PFL
6	TEF1 Δ ACC1/PFL double mutant of WT	TEF1 Δ ACC1/PFL
7	Δ FAA2/ TEF1 Δ ACC1 double mutant of WT	Δ FAA2/ TEF1 Δ ACC1
8	Δ FAA2/ TEF1 Δ ACC1/PFL triple mutant of WT	Δ FAA2/ TEF1 Δ ACC1/PFL
9	<i>Candida tropicalis</i> – Wild type	CT -WT
10	<i>Pachysolen tannophilus</i> – Wild type	PT - WT

6.0.2 Profile of fatty acids produced by yeast strains of the invention provided with 20% Glucose as carbon source.

10 Yeast strains were grown on the defined growth medium given in Table 5 (Modified Wickerham Synthetic Medium), where the selected sole carbon source was 20% Glucose. The growth of the yeast strains, under fermentation growth conditions, and the subsequent extraction and analysis of extracellular lipids produced (fatty acid production) were as defined in

15 Example 2.0.0. The extracted fatty acids were concentrated to a volume 2 ml and was sent for GC-MS analysis. The profile of fatty acids in the 2ml volume is set out in Table 15 and Figure 19.

Table 15: Profile of fatty acids produced by yeast strains on 20% glucose

20

Strain	C:6	C:8	C:11	C:12	C:14	C:14,1	C:15	C:16	C:17,1	C:18	C:20	C>20	Total
1	-	--	--	-	-	-	-	14%	-	11%	0	52%	77%
2	-	--	--	-	-	-	5%	13%		42%	9.5%	0	69.5%
4	1.4%	1.9%	2.9%		2%	14.8%	-	34.1%	-	30%	2.1%	-	89.2%
5	-	-	-	-	-	14%	-	49.5%	-	30%	2.5%	-	96%
6	-	-	-	-	2.1%	-	-	52%	-	26.5%	-	2.8%	83.4%
7	2.4%	-	-	2.9%		14.7%	-	41%	13.3%	14.6%	3.2%	--	92.1%
8	3.6%	5%	-	-	4.1%	21.1%	-	30%	16.2%	12.6%	-	-	92.6%
9	-	-	-	-	-	-	-	35%	-	22%	-	-	57%

Figure 19 shows that when the strains are grown on 20% glucose as substrate, the strains 4, 5 (comprising the PFL system which increases cytosolic levels of acetyl coA) enhances the yield of fatty acids being produced from glucose. Further the triple mutant (strain 8) shows that the pathway engineering of fatty acid biosynthesis process has a positive effect on the overall yield of fatty acids.

Table 15 reveals the effect of mutations on the profile of fatty acids produced. The bulk of fatty acids produced by WT yeast strain 1 are very long chain fatty acid (VLCFA) group, not suitable for biodiesel production. All of the mutations have dramatic and unexpected effect in reducing the chain-length of fatty acids while grown on glucose. Most of the fatty acids secreted by the mutant strains are medium-chain fatty acids which are commercially important in their role as biodiesel. The effect of FAA2 deletion alone on chain-length of fatty acids was unexpected.

Further, the double mutants in strains 4, 5, 6 and 7, produce fatty acids comprising an increasing proportion of shorter chain-length fatty acids.

The triple mutant (strain 8) shows lesser proportion of longer stearic acid and higher proportion of shorter chain-length fatty acids. The presence of these shorter fatty acids makes the quality of biodiesel better as the shorter fatty acids burn more completely and do not freeze in lower temperatures.

6.0.3 Profile of fatty acids produced by yeast strains of the invention provided with 5% pure glycerol as carbon source.

Yeast strains were grown on the defined growth medium given in Table 5 (Modified Wickerham Synthetic Medium), where the selected sole carbon source was 5% pure glycerol. The growth of the yeast strains, under fermentation growth conditions, and the subsequent extraction and analysis of extracellular lipids produced (fatty acid production) were as defined in Example 2.0.0. The extracted fatty acids were concentrated to a volume 2 ml and was sent for GC-MS analysis. The profile of fatty acids in the 2ml volume is set out in Table 16 and Figure 20.

Table 16: Profile of fatty acids produced by yeast strains on 5% pure glycerol

Strain	C:6	C:8	C:14	C:14,1	C:16	C:18	C:20	C >20	C:18-1	C:18-2,6	Total
1								0	74%		74%
2			4%		18%	30.5%		0			52.5%
3.					1%	1%			7%	2%	11%
5			2%	20%	43%	32%	2.6%				99.6%
6				23.4%	45%	31.4%					99.8%
7	5.8%	1%			20%	11.5%					38.3%
8	10%			5.1%	7.8%	7.2%					30.2%
9						0		2,25%			2.25%
10					4%	0%			15%	0	19%

Figure 16 shows that when the strains are grown on 5% pure glycerol, where the single mutation strains (strain 2 and 3) produce lower levels of fatty acids than the WT strain 1. However, the double mutants comprising the PFL system show enhanced yields of fatty acids. The probable reason for this is that an extra NADH which is generated while incorporating glycerol into glycolytic pathway (glycerol >> glyceraldehydes-3-phosphate) remains unused, thereby creating a NAD⁺ imbalance which hampers the growth of single mutants. However, introduction of PFL system relieves the cellular

system of this NADH imbalance and thus, we see better performance by double mutant strains with PFL system.

Table 16 reveals the effect of mutations on the profile of fatty acids produced. The double mutants (strains 5 and 6) comprising the PLF system show similar fatty acid profile consisting of MCFAs. The double mutant lacking the PFL system (strain 7) fails to mimic the high fatty acid yields of strain 5 and 6, confirming the key role of PFL system in enabling fermentation on glycerol to yield fatty acids. The triple mutant, again shows the surprising advantage of producing a higher proportion of lower chain-length fatty acids, although the total amount of fatty acids produced is less than WT or double mutant strains.

6.0.4 Profile of fatty acids produced by yeast strains of the invention provided with 5% crude glycerol as carbon source

Yeast strains were grown on the defined growth medium given in Table 5 (Modified Wickerham Synthetic Medium), where the selected sole carbon source was 5% crude glycerol. The growth of the yeast strains, under fermentation growth conditions, and the subsequent extraction and analysis of extracellular lipids produced (fatty acid production) were as defined in Example 2.0.0. The extracted fatty acids were concentrated to a volume 2 ml and was sent for GC-MS analysis. The profile of fatty acids in the 2ml volume is set out in Table 17 and Figure 21.

Table 17: Profile of fatty acids produced by yeast strains on 5% crude glycerol

Strain	C:6	C:8	C:10	C:12	C:14	C:14, 1	C:16	C:17	C:18	C >20	C:18- 9	C:18- 9,12	Total
1.							4,5		7,7		13,3	3,5	29%
2.							3.5 %		1.57 %		63.5 %	17.6%	86.17 %
3.							4%		1.8%		65%	17.84 %	88.64 %

4.	6.2 %	1.2 %					14%		5.4%				26.8%
5.	9.5 %					3.8%	6.8 %		2.7%				22.8%
7.	7%					3.4%	7.1 %		4.1%				21.6%
8.	5.2 %		4.9 %	1.2 %	12.9 %		8.7 %	4.4 %	1.5%	1.5 %			40.3%
9							1,29		0		0	0	1.29%
10							2.7 %		13%		53.5 %		69.2

Crude glycerol includes other components that may either promote or inhibit growth and fermentation of the yeast strains. The single mutant strains 2 and 3 as well as the triple mutant 8 show the capacity to grow on crude glycerol and to produce more MCFAs than the WT strain. Strain 10, is also surprisingly robust in its capacity to grow and ferment crude glycerol to fatty acids. The importance of deletion of the FAA2 gene, and the activation of ACC1 gene for fatty acid production by fermentation on glycerol is also confirmed in the performance of strains 2, 3 and 8.

10

6.0.5 Profile of fatty acids produced by yeast strains of the invention provided with on 15% xylose as carbon source

Yeast strains were grown on the defined growth medium given in Table 5 (Modified Wickerham Synthetic Medium), where the selected sole carbon source was on 15% xylose. The growth of the yeast strains, under fermentation growth conditions, and the subsequent extraction and analysis of extracellular lipids produced (fatty acid production) were as defined in Example 2.0.0. The extracted fatty acids were concentrated to a volume 2 ml and was sent for GC-MS analysis. The profile of fatty acids in the 2ml volume is set out in Table 18 and Figure 22.

20

Table 18: Profile of fatty acids produced by yeast strains on 15% Xylose

Strain	C:6	C:8	C:10	C:11	C:12	C:14	C:14,1	C:16	C:16,1	C:17,1	C:18	C:20	Total
1.		2.1%			2.2%	2.3%	9.9%	16.7%		29.9%	5.4%	2.3%	70.8%
2	1%	9.7%	2.2%					2.2%				9.4%	24.5%
3				2,70%		2.4%	17.9%	43.3%	3.1%		15%	2.5%	86.9%
4	2.3%	1.1%		2%			10%	29.7%			26.3%	1.7%	73.1%
5.							17%	50%		19.6%		5%	91.6%
6.								28%		10%	10%		48%
8						2.14%	8.9%	39%		12.7%	14.2%	2.3%	79.2%

Surprisingly, the strains 3, 4 5, and 8 were out-performed the WT in utilizing xylose, and producing fatty acids. Again the PFL system contributes to this enhanced capacity to produce fatty acids in the single, double and triple mutant strains.

6.0.6 Profile of fatty acids produced by yeast strains of the invention provided with on hydrolysed Wheat lignocellulose as Carbon source.

Yeast strains were grown on the defined growth medium given in Table 5 (Modified Wickerham Synthetic Medium), where the selected sole carbon source was on hydrolysed wheat lignocellulose (added as 10ml/liter growth medium). The growth of the yeast strains, under fermentation growth conditions, and the subsequent extraction and analysis of extracellular lipids produced (fatty acid production) were as defined in Example 2.0.0. The extracted fatty acids were concentrated to a volume 2 ml and was sent for GC-MS analysis. The profile of fatty acids in the 2ml volume is set out in Table 19 and Figure 23.

Table 19: Profile of fatty acids produced by yeast strains on hydrolysed wheat lignocellulose

Strain	C:6	C:8	C:10	C:12	C:14	C:14,1	C:16	C:17	C:18	C >20	C:18-9	C:18-9,12	Total
1							4,5		7,7		13,3	3,5	29%
2							1.5		2,4		27%	7%	37.9%
3.							0		0		2,5	0	2.5%
4.	6.2%	1.2%					14%		5.4%				26.8%
5.	9.5%					3.8%	6.8%		2.7%				22.8%
8.	5.2%		4.9%	1.2%	12.9%		8.7%	4.4%	1.5%	1.5%			40.3%
9							1,29		0		0	0	1.29%
10									5%		1.5%		6.25%

5

The growth of the engineered yeast strains on digested lignocelluloses in the form of a wheat straw hydrolysate showed that the triple mutant strain (strain 8) produced more fatty acids than other engineered strains, while the single mutant FAA2 was also effective. Strain 8 however, has the additional advantage, compared to strain 2, in producing an even distribution of fatty acids of medium chain fatty acid range, important for biodiesel of the best quality. Furthermore, all of the fatty acids secreted by strain 8 are aliphatic, saturated fatty acids, while unsaturated fatty acids are distinctly present in the fatty acid profile of strain 2.

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Applicant's or agent's file reference P81000119PCT	International application No. PCT/EP2011/060237
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**INDICATIONS RELATING TO DEPOSITED MICROORGANISM
OR OTHER BIOLOGICAL MATERIAL**

(PCT Rule 13bis)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page <u>33</u> , line <u>11</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution CBS Centraalbureau voor Schimmelcultures	
Address of depositary institution (including postal code and country) Uppsalalaan 8 NL-3584 CT Utrecht The Netherlands	
Date of deposit 26 April 2010	Accession Number CBS 126804
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
All Designations	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

<p align="center">For receiving Office use only</p> <p><input checked="" type="checkbox"/> This sheet was received with the international application</p> <hr/> <p>Authorized officer Koestel, Gilbert</p>	<p align="center">For International Bureau use only</p> <p><input checked="" type="checkbox"/> This sheet was received by the International Bureau on: 27 June 2011 (27.06.2011)</p> <hr/> <p>Authorized officer Nathalie WAGNER</p>
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EPO - DG 2

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Claims:

1. A genetically modified microorganism for the extracellular production of free fatty acids and esters thereof, wherein said microorganism characterised by:
 - 5 a. reduced fatty acyl-coA synthetase activity conferred by a FAA2 gene deletion, and
 - b. enhanced acetyl CoA carboxylase expression conferred by a recombinant endogenous ACC1 gene wherein said gene is operably linked to a heterologous promoter, and
 - 10 c. a transgene encoding a pyruvate formate lyase comprising PflA and PflBwherein said microorganism is selected from among species of *Aspergillus*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Fusarium*, *Lindnera*, *Lipomyces*, *Monascus*, *Mucor*, *Pachysolen*, *Pichia*,
15 *Rhizopus*, *Rhodospiridium*, *Rhodotorula*, *Saccharomyces*, *Schizosaccharomyces*, *Trichosporon*, *Yarrowia*, and *Zygosaccharomyces*.
2. A genetically modified microorganism of claim 1, further characterised by a transgene encoding an acyl CoA-ACP thioesterase, wherein said
20 thioesterase is selected from among: Soyabean (*Glycine max*); *Chlamydomonas reinhardtii* (Protein ID - A8HY17; SEQ ID NO: 53); *Arabidopsis thaliana* (Protein ID - Q9SJE2; SEQ ID NO: 54); *Ricinus communis* (Protein ID - B9RAC3; SEQ ID NO: 56); *Triticum aestivum* (GeneID: 543005); CtFatA from *Brassica napus* (Protein ID - Q43745; SEQ ID NO: 58)); CtFatA from *C. tinctorius* (Protein ID - Q42715; SEQ ID
25 NO: 60); GmFatA1 from *G. mangostana* (Protein ID - O04792; SEQ ID NO: 62); CwFatB1 from *C. hookeriana* (Protein ID - Q39513; SEQ ID NO:

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64); CwFatB1 from *C. wrightii* (Protein ID - Q39662; SEQ ID NO: 66);
GmFatB1 from *G. Mangostana* (Protein ID - O04794; SEQ ID NO: 68).

3. A genetically modified microorganism of claim 1 or 2, further
characterised by enhanced expression of an acyl-coenzymeA:ethanol
5 O-acyltransferase conferred by:

a. a recombinant endogenous acyl-coenzymeA:ethanol O-
acyltransferase (EEB1) gene wherein said gene is operably
linked to a heterologous promoter.

4. A genetically modified microorganism of any one of claims 1-3, further
10 characterised by a transgene encoding a heterologous cytosolic Acyl
CoA thioesterase (CTE-1), wherein said CTE-1 is selected from
among: *Mus musculus* (Protein ID - O55137; SEQ ID NO: 74);
Arabidopsis thaliana (Protein ID - Q5FYU1; SEQ ID NO: 76) or *Rattus
norvegicus* (Protein ID - Q6AZ44; SEQ ID NO: 78)

15 5. A genetically modified microorganism of any one of claims 1-4, further
characterised by one or more transgene encoding a heterologous
glycerol kinase or a xylose isomerase or both; wherein said glycerol
kinase is selected from among Glycerol Kinase from *Saccharomyces
cerevisiae* (Protein ID - P32190; SEQ ID NO: 92), and said xylose
20 isomerase is selected from among Xylose isomerase from *Clostridium
phytofermentas* (Protein ID - A9KN98; SEQ ID NO: 88); *Yersinia
pestis* – (Protein ID - Q8Z9Z1; SEQ ID NO: 90).

6. A genetically modified microorganism of any one of claims 1-5, further
characterised by deletion of an endogenous formate dehydrogenase
25 FDH gene wherein said FDH gene is selected from among: GeneID:
854570 (SEQ ID NO: 44); GeneID: 8300341 (SEQ ID NO: 46); and
GeneID: 2907923 (SEQ ID NO: 48).

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7. A genetically modified microorganism of claim 6, further characterised by a transgene encoding a heterologous formate hydrogen lyase, where said lyase is *E.coli* formate hydrogen lyase (Protein ID - C8UET5; SEQ ID NO: 51)
- 5 8. A genetically modified microorganism of any one of claims 1-7, further characterised by the deletion of an endogenous alcohol dehydrogenase (ADH) gene, wherein said ADH gene is selected from among: GeneID: 854068 (SEQ ID NO: 79); GeneID: 2538902 (SEQ ID NO: 81); GeneID: 2868277 (SEQ ID NO: 83); and GeneID: 852442 (SEQ ID NO: 85).
- 10 9. A growth medium comprising the following components:

Component	Composition (per litre medium)
Carbon Source	
carbon source	≥50g
Nitrogen Source	
Ammonium sulphate	5g
Vitamins*	
Biotin	20 µg
Folic acid	2 µg
Inositol	10 mg
Niacin	400 µg
Riboflavin	200 µg
Pyridoxine hydrochloride	400 µg
Thiamine hydrochloride	400 µg
Compounds supplying trace elements	
Boric acid	500 µg
Copper sulphate	40 µg
Potassium iodide	100 µg
Ferric chloride	200 µg
Manganese sulphate	400 µg
Sodium molybdate	200 µg
Zinc sulphate	400 µg
Salts	
Potassium phosphate monobasic	900 mg
Potassium phosphate dibasic	200 mg

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Magnesium sulphate	500 mg
Sodium chloride	100 mg
Calcium chloride	100 mg

and wherein said carbon source is selected from at least one of glucose, glycerol, xylose, hydrolysed cellulose and hemicellulose, starch, sugar alcohol and xylan.

5 10. Method for the extracellular production of free fatty acids and esters thereof, comprising the steps of:

- 10
- a. introducing a genetically modified microorganism according to any one claim 1-8 into a growth medium to produce a culture;
 - b. incubating said culture, wherein the growth medium in said culture is aerated with oxygen;
 - c. further incubating said culture after step (b) in the absence of an oxygen supply;
 - d. recovering an extracellular phase comprising free fatty acids and esters from said culture;

15 wherein said microorganism is a yeast or fungal strain further characterised in that the genetic modification of said microorganism results in enhanced production of fatty acids and their esters of shorter chain-length.

20 11. The method of claim 10, where the microorganism is selected from *Candida tropicalis*, and *Pachysolen tannophilus*.

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12. The method of claim 10 or 11 , where the growth medium comprises a carbon source selected from at least one of glucose, glycerol, xylose, hydrolysed cellulose and hemicellulose, starch, sugar alcohol and xylan.

13. The method of claim 12, wherein the growth medium comprises or
5 consists of the components set out in claim 9.

14. Use of a yeast or fungal strain for the extracellular production of free fatty acids and esters thereof, where the yeast or fungal strain is a genetically engineered microorganism characterised by:

10 a. reduced fatty acyl-coA synthetase activity conferred by a FAA2 gene deletion;

b. enhanced acetyl CoA carboxylase expression conferred by a recombinant endogenous ACC1 gene wherein said gene is operably linked to a heterologous promoter, and

15 c. a transgene encoding a pyruvate formate lyase comprising PflA and PflB

20 wherein said microorganism is selected from among species of *Aspergillus*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Fusarium*, *Lindnera*, *Lipomyces*, *Monascus*, *Mucor*, *Pachysolen*, *Pichia*, *Rhizopus*, *Rhodospiridium*, *Rhodotorula*, *Saccharomyces*, *Schizosacchomyces*, *Trichosporon*, *Yarrowia*, and *Zygosacchomyces*, characterised in that said the genetic modification of said microorganism results in enhanced production of fatty acids and their esters of shorter chain.

25 15. The use of claim 14, where the microorganism is selected from *Candida tropicalis*, and *Pachysolen tannophilus*.

Figure 1a

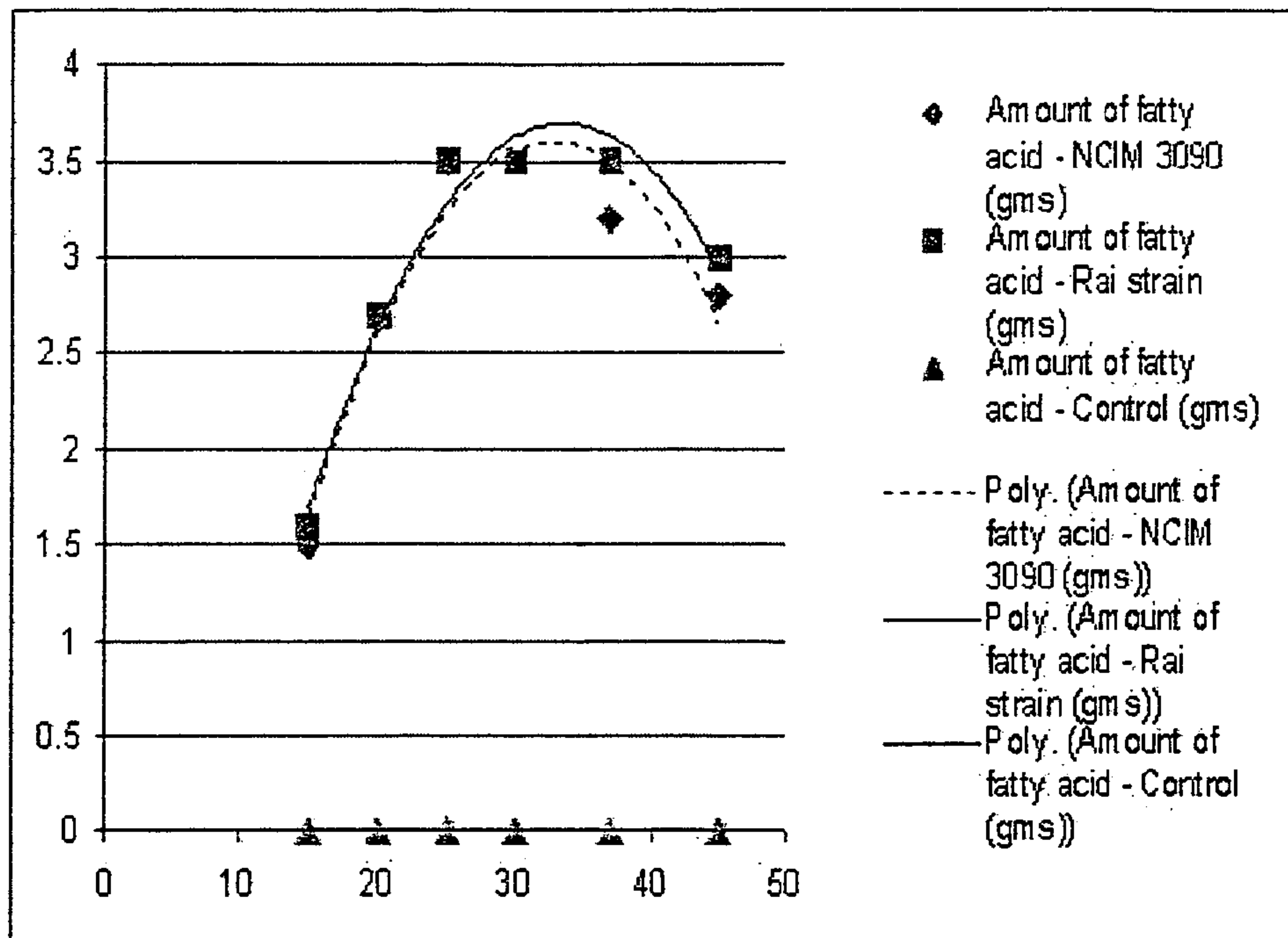


Figure 1b

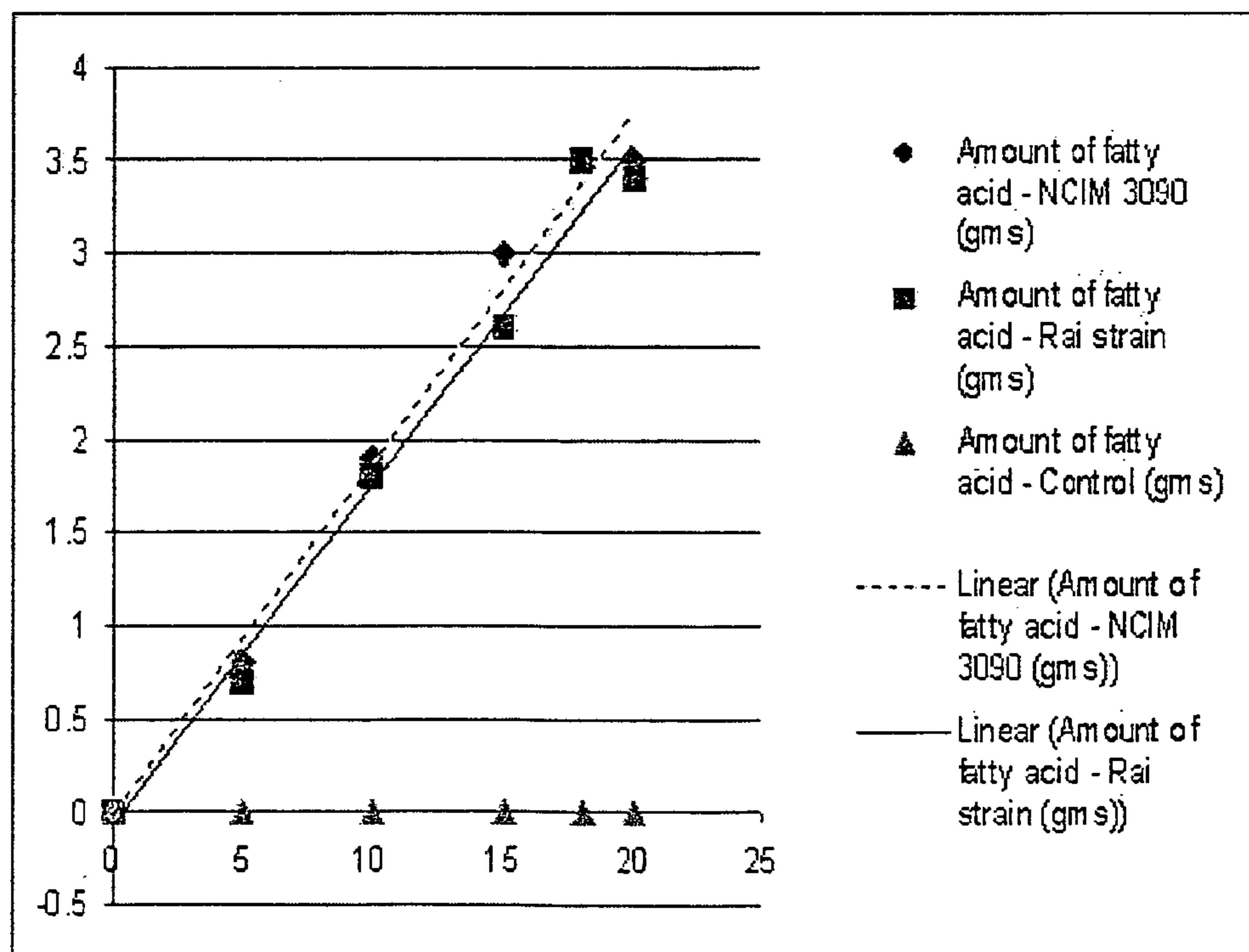


Figure 1c

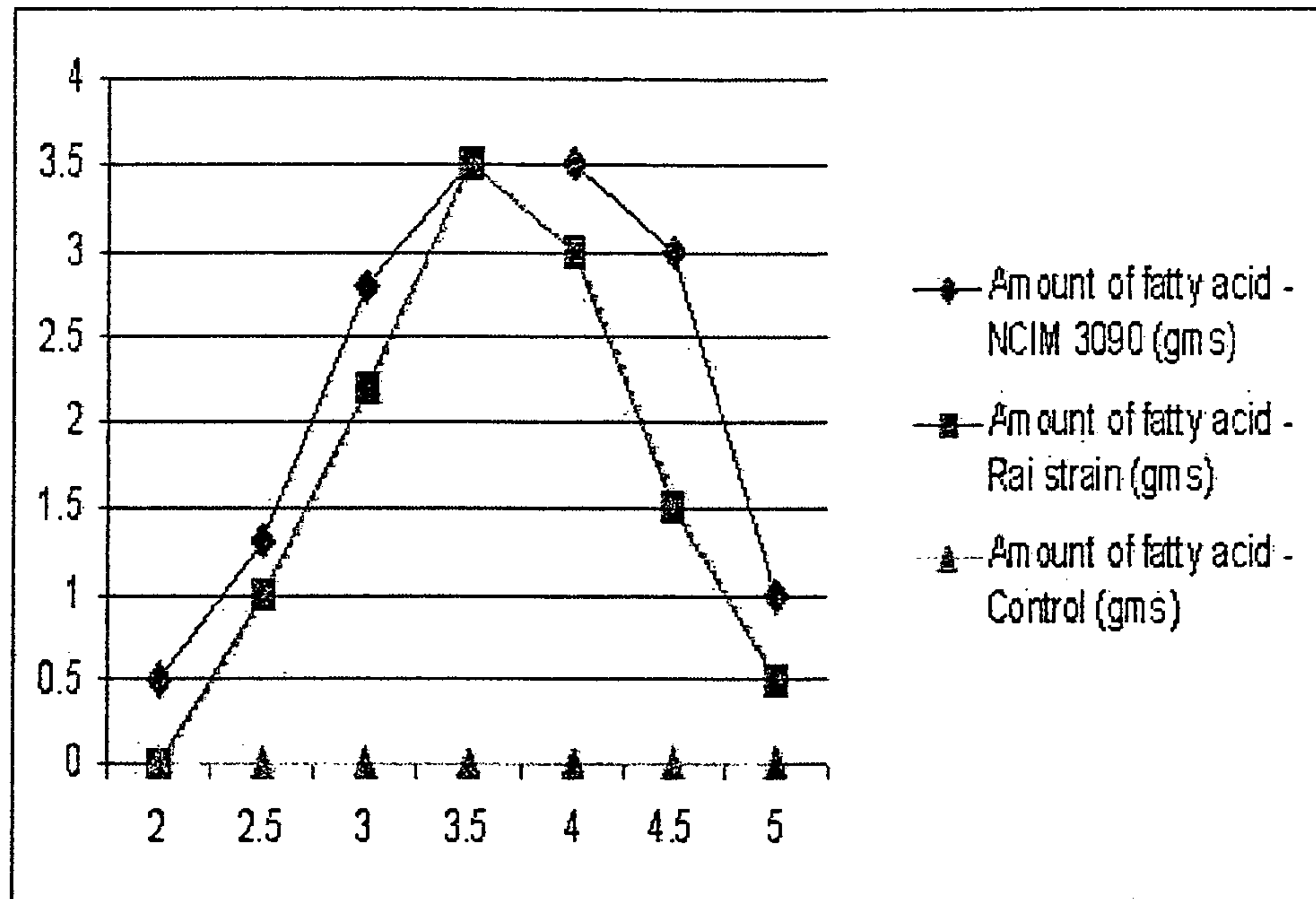
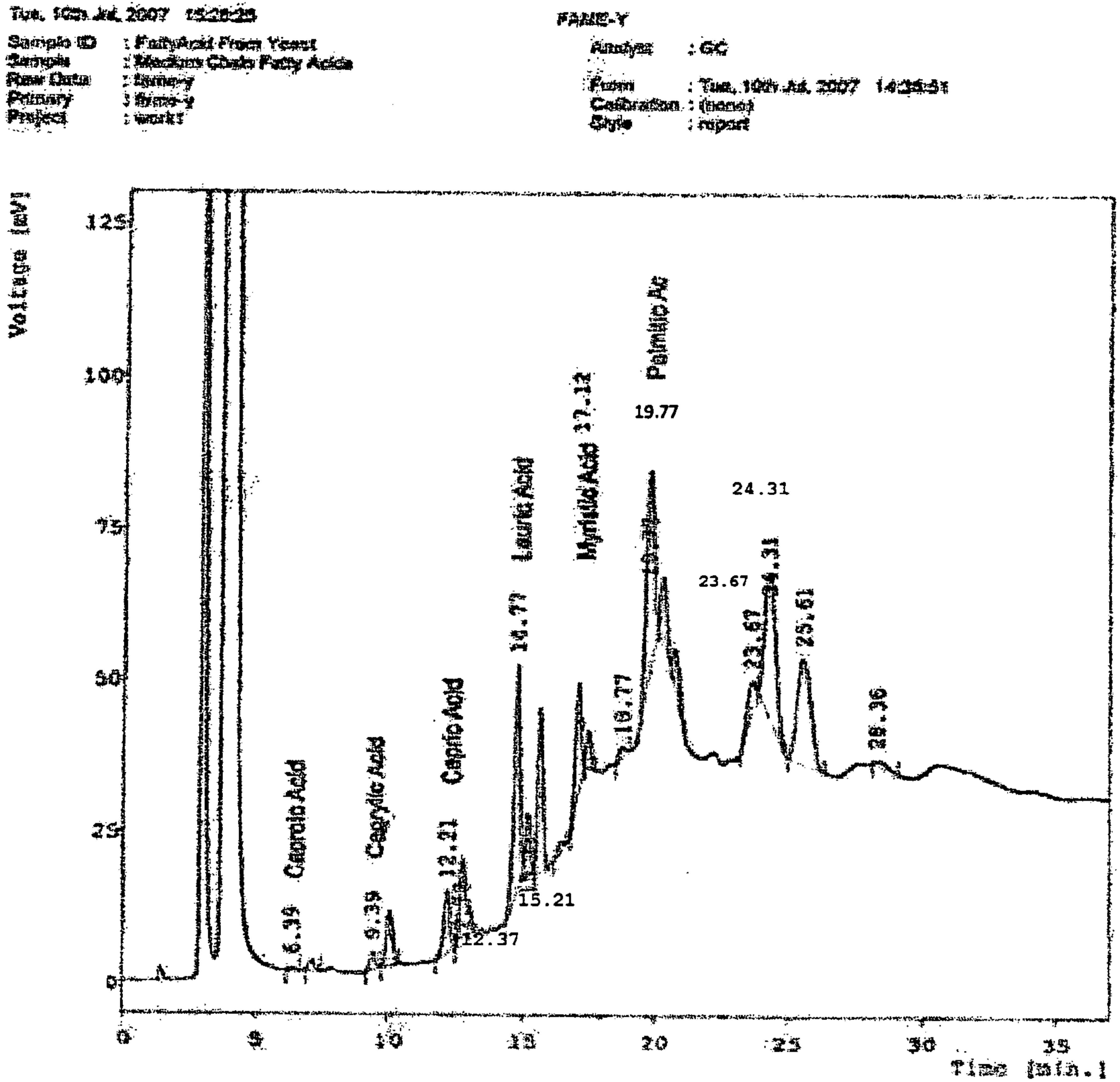


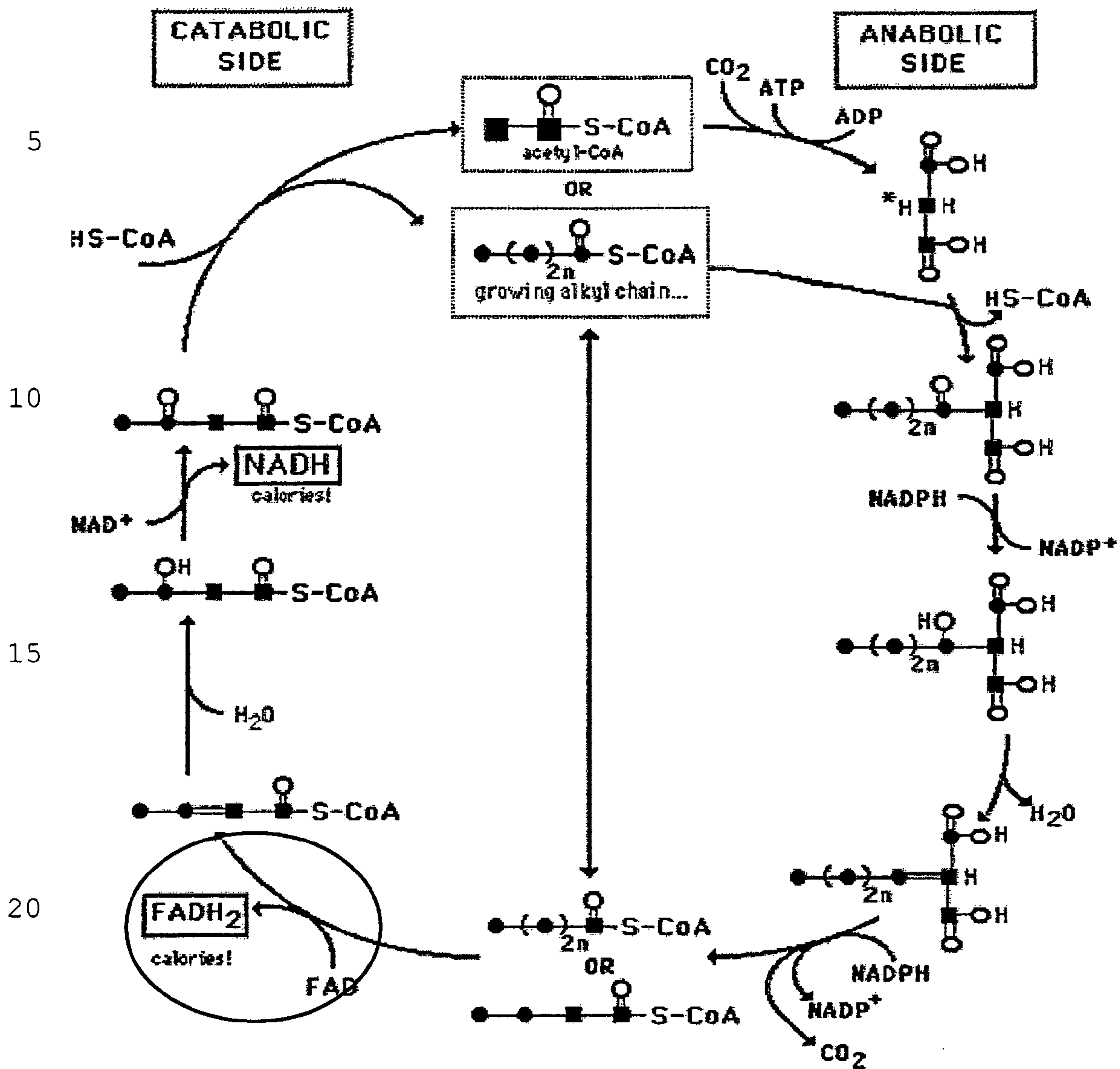
Figure 2



Result Table - Calculation Method Uncal

Peak No.	Retain. time	Area [mV.s]	Height [mV]	NDS [min.]	Area [%]	Height [%]
1	6.387	5.4011	0.359	0.253	0.124	0.153
2	7.140	25.4164	1.706	0.240	0.582	0.727
3	9.393	40.9519	2.783	0.240	0.936	1.186
4	10.060	129.5674	9.039	0.233	2.963	3.254
5	12.207	162.2671	10.256	0.253	3.716	4.373
6	12.787	265.4293	14.223	0.267	6.079	6.064
7	14.773	359.4489	30.779	0.233	11.513	15.661
8	15.213	135.8003	9.220	0.193	2.421	3.531
9	15.647	347.6674	27.072	0.213	7.963	11.543
10	16.453	18.3387	1.346	0.233	0.420	0.575
11	17.120	283.1979	17.757	0.240	6.028	7.571
12	17.500	74.0039	6.113	0.207	1.695	2.605

Figure 3a



Deletion of FAA2 -
Downregulation of fatty
acid oxidation pathway

Figure 3b

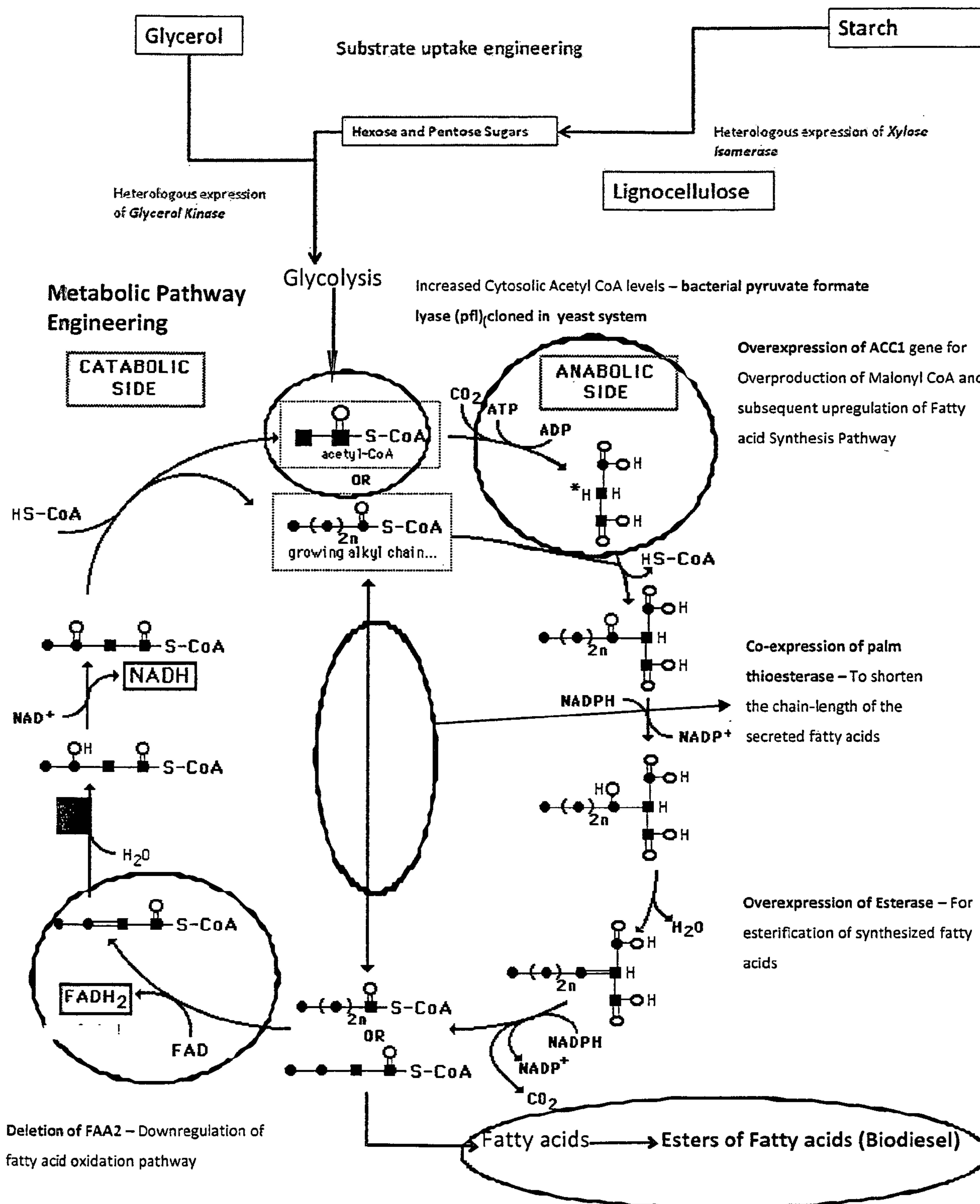


Figure 4

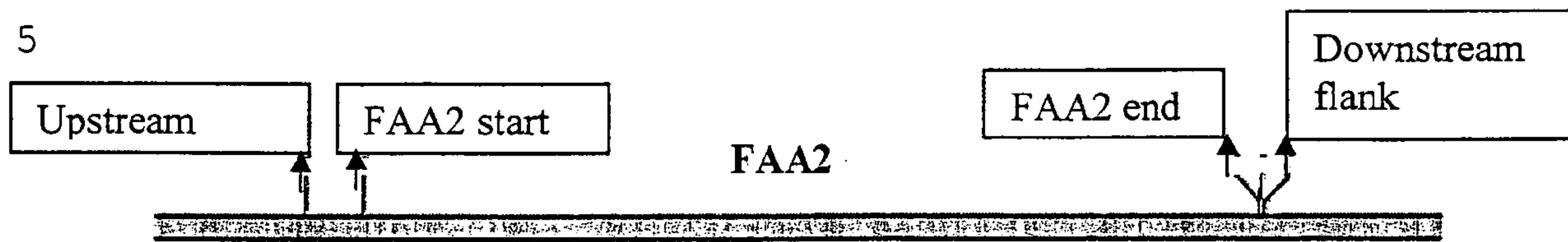


Figure 5

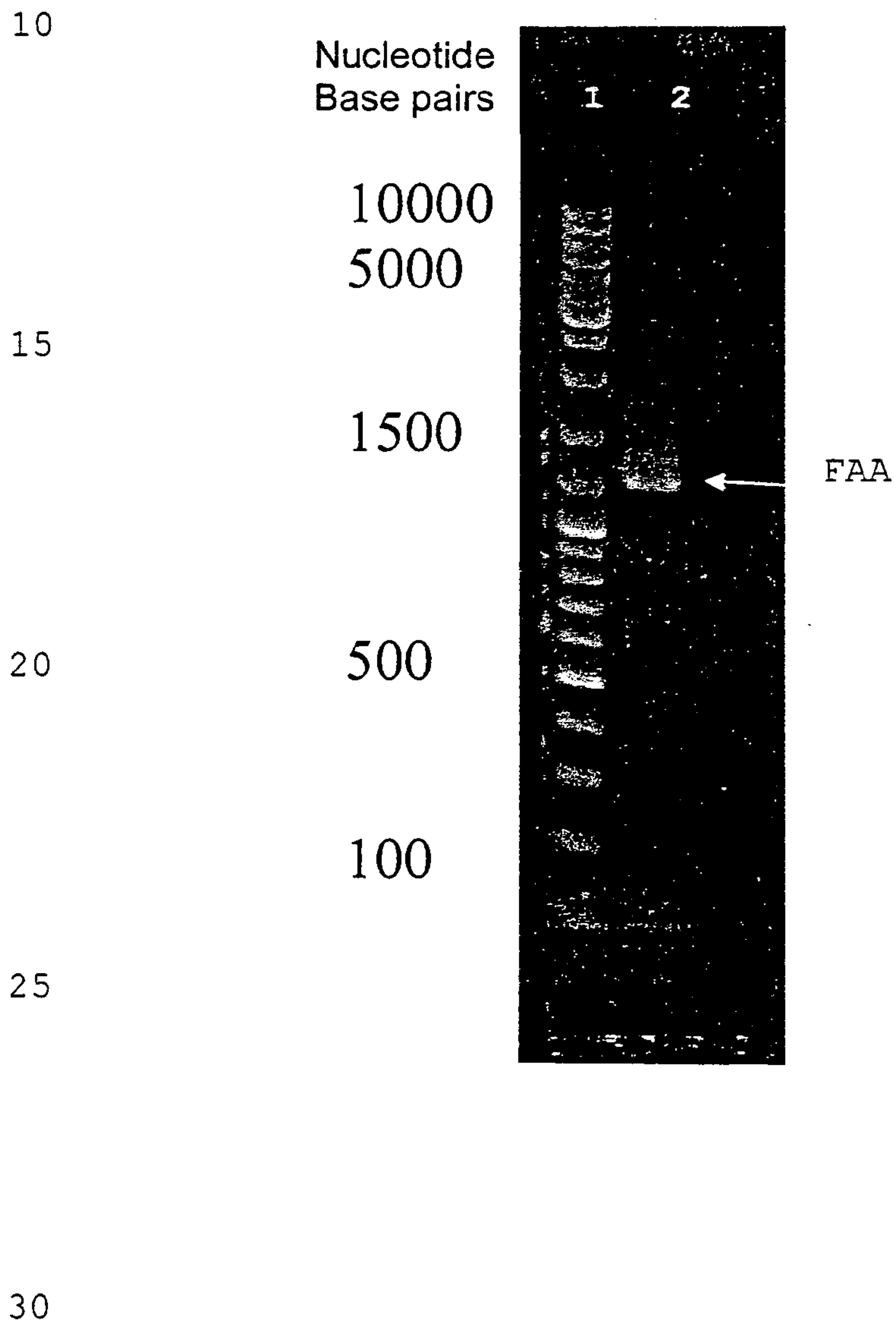
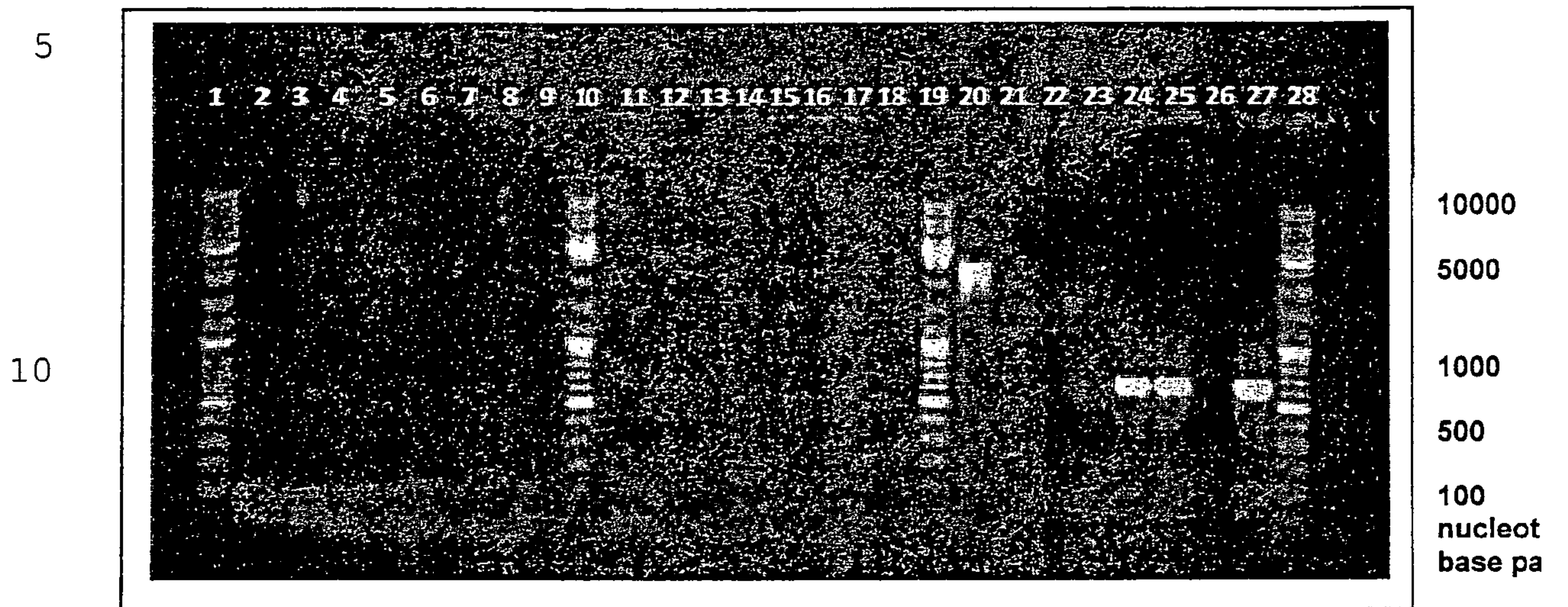
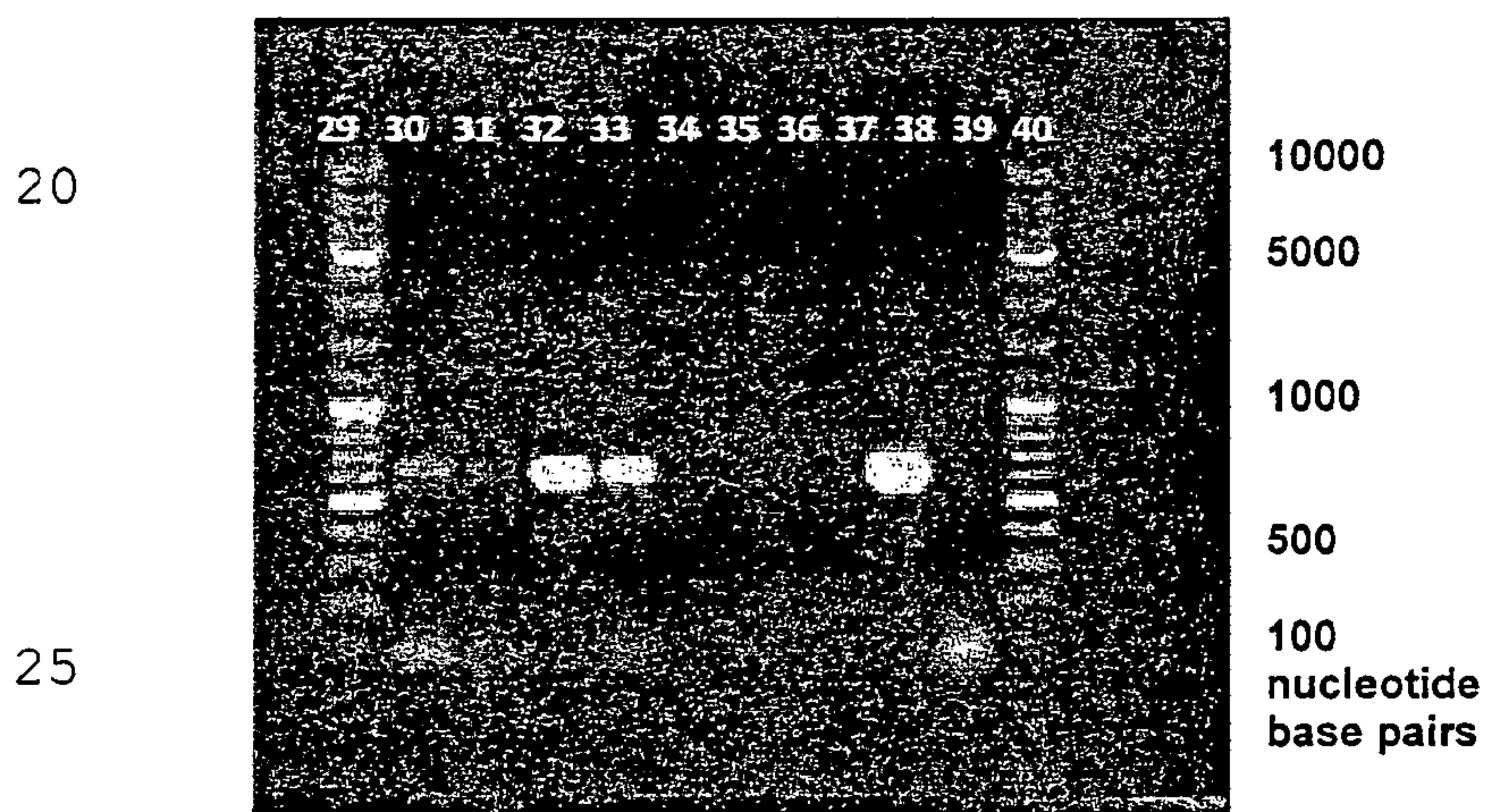


Figure 6a



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Figure 6b



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Figure 7

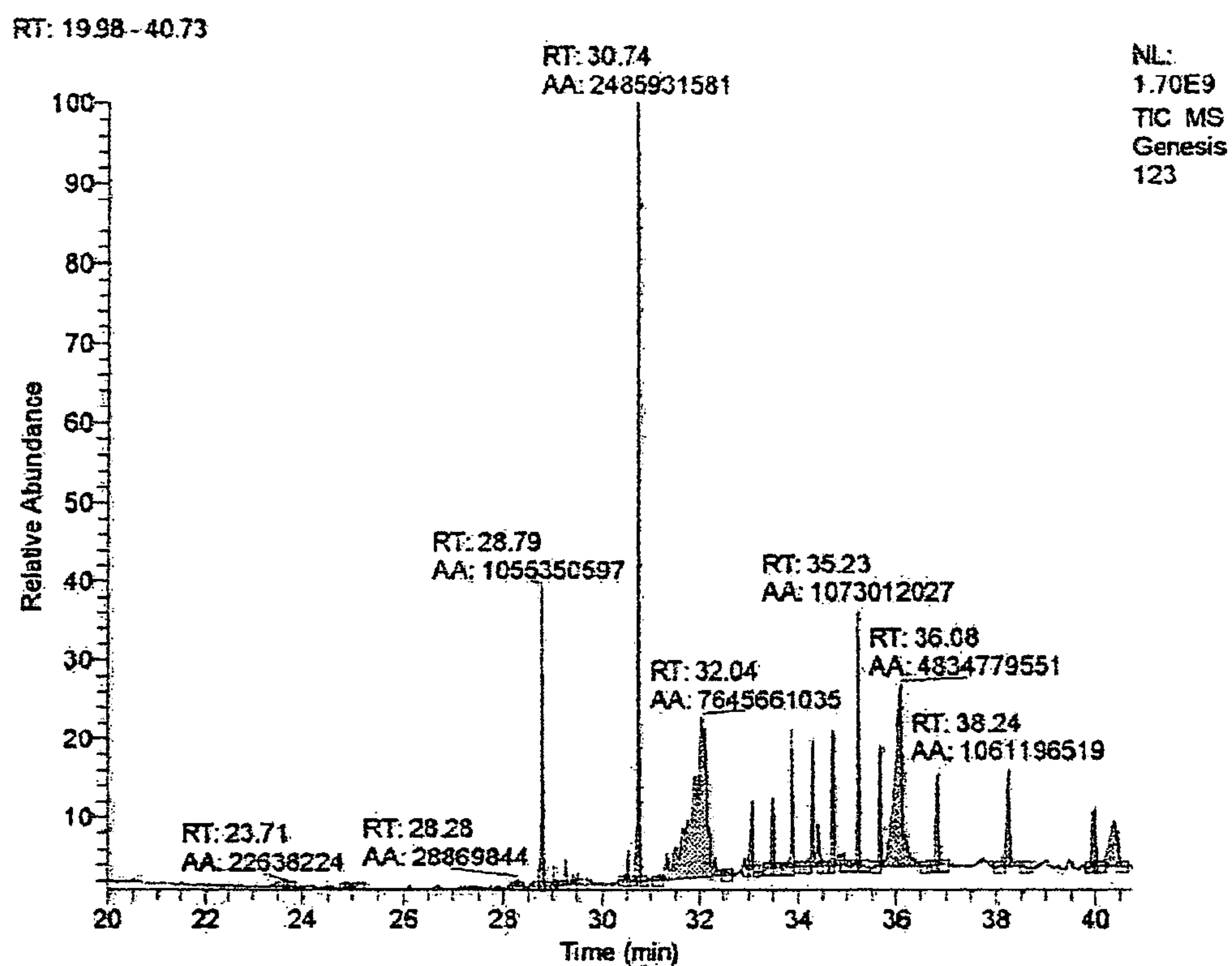
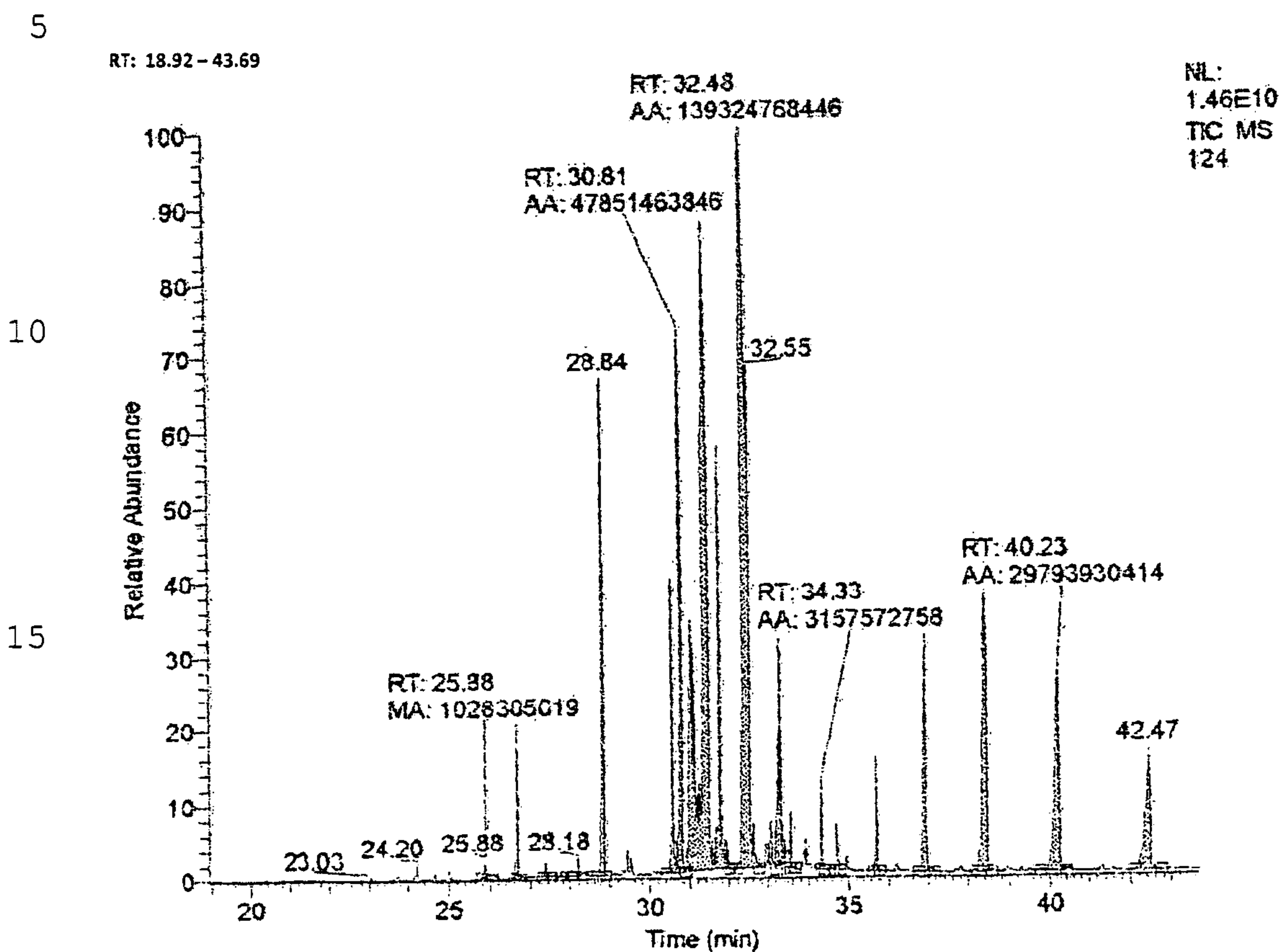


Figure 8



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Figure 9

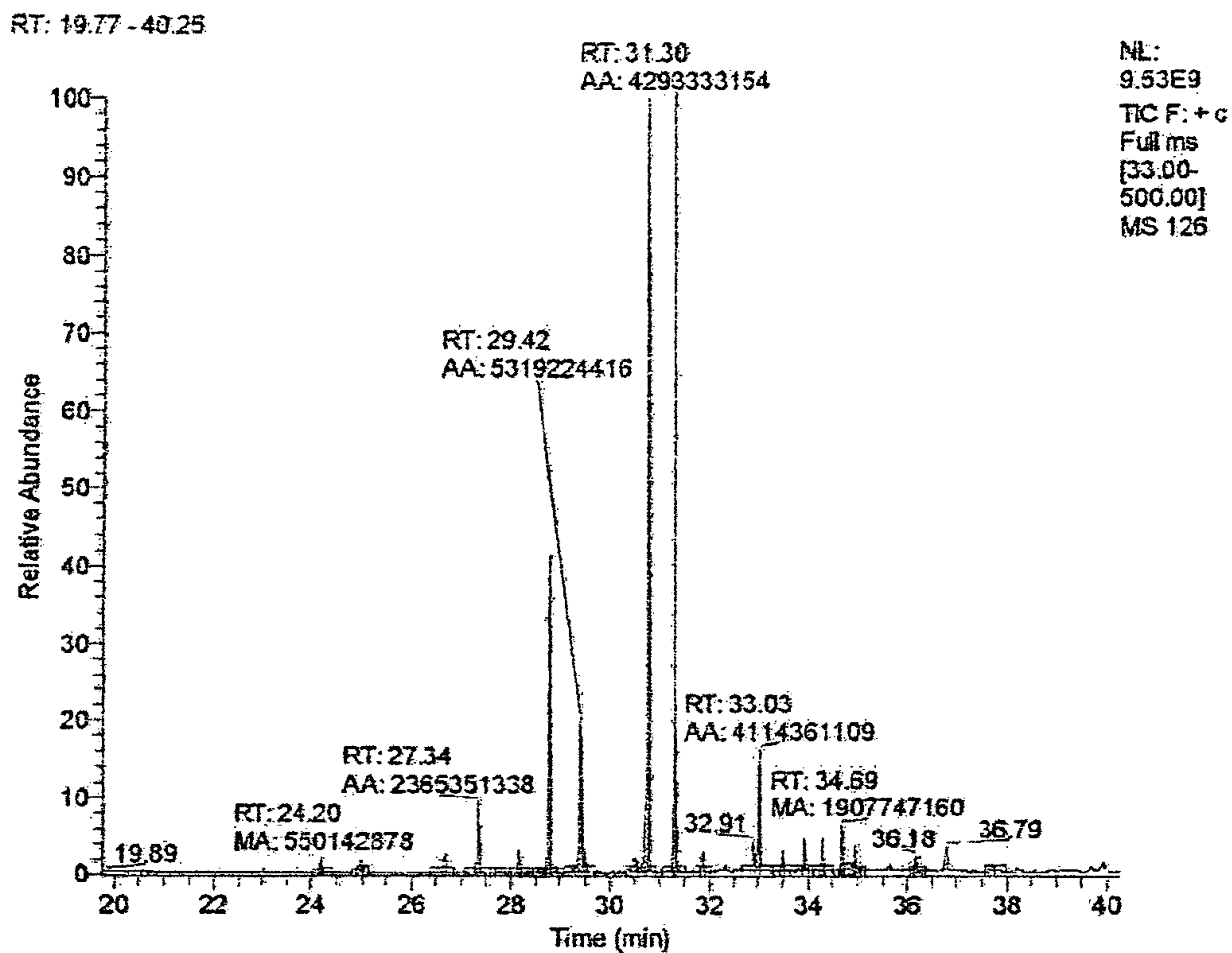


Figure 10

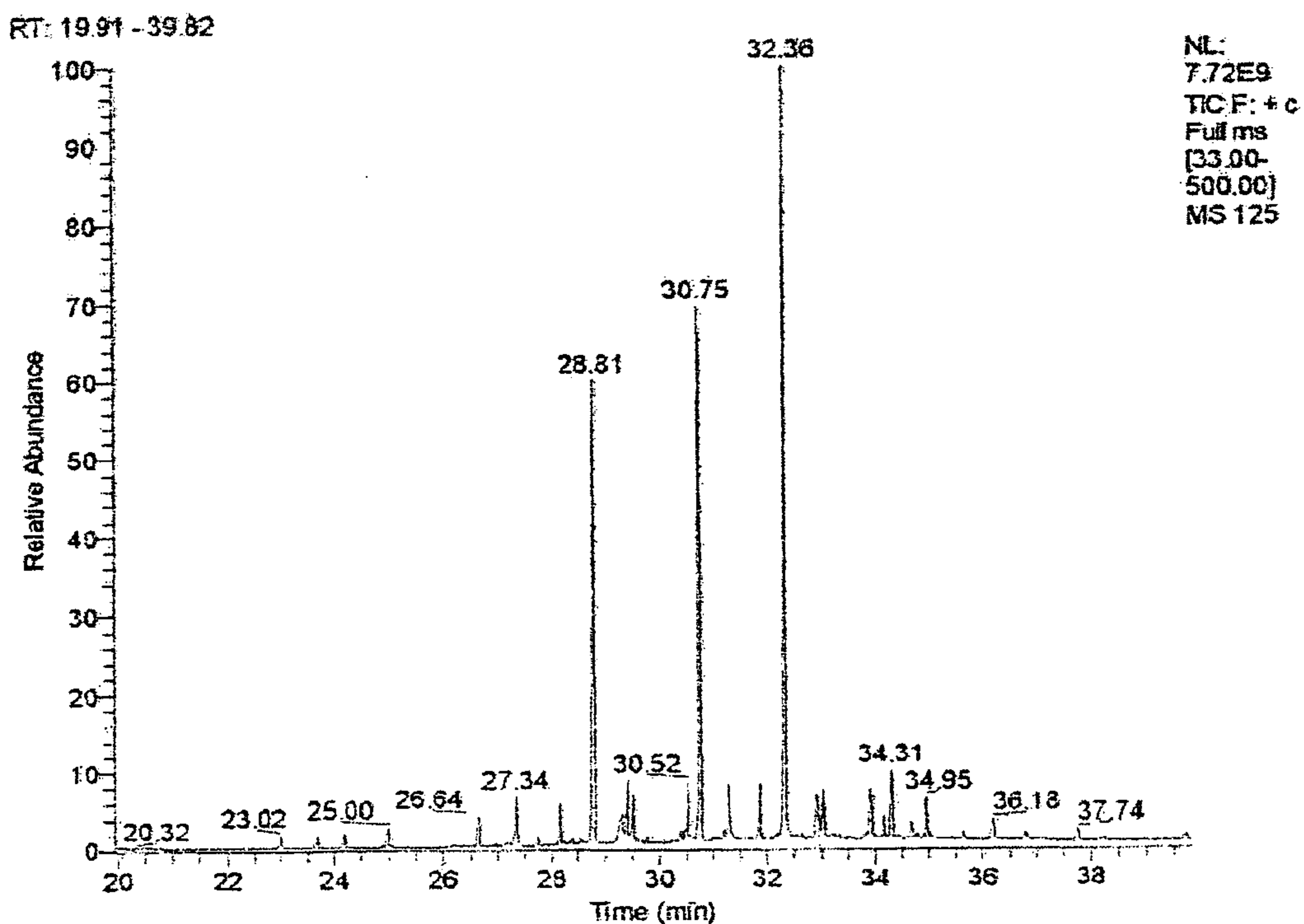


Figure 11

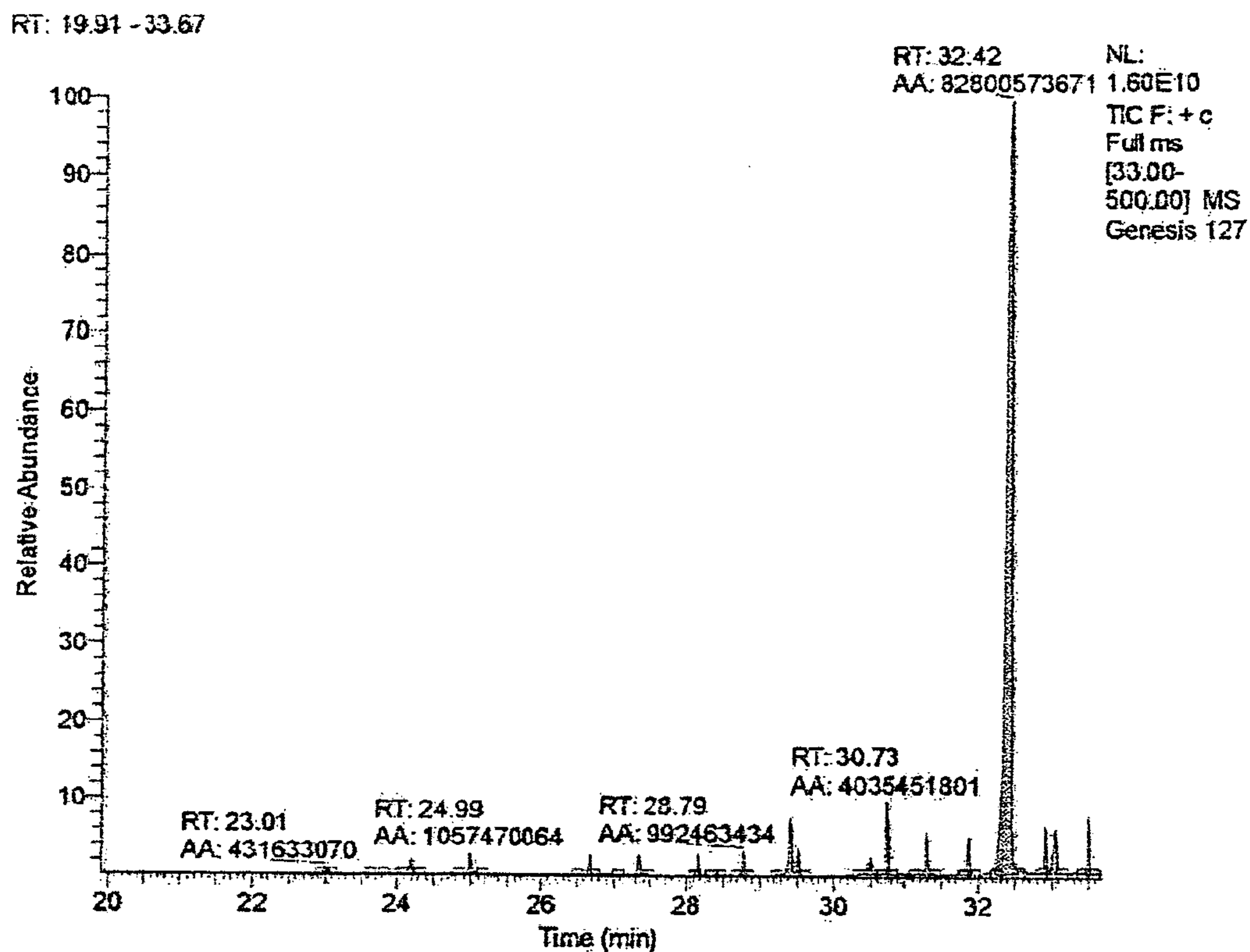


Figure 12

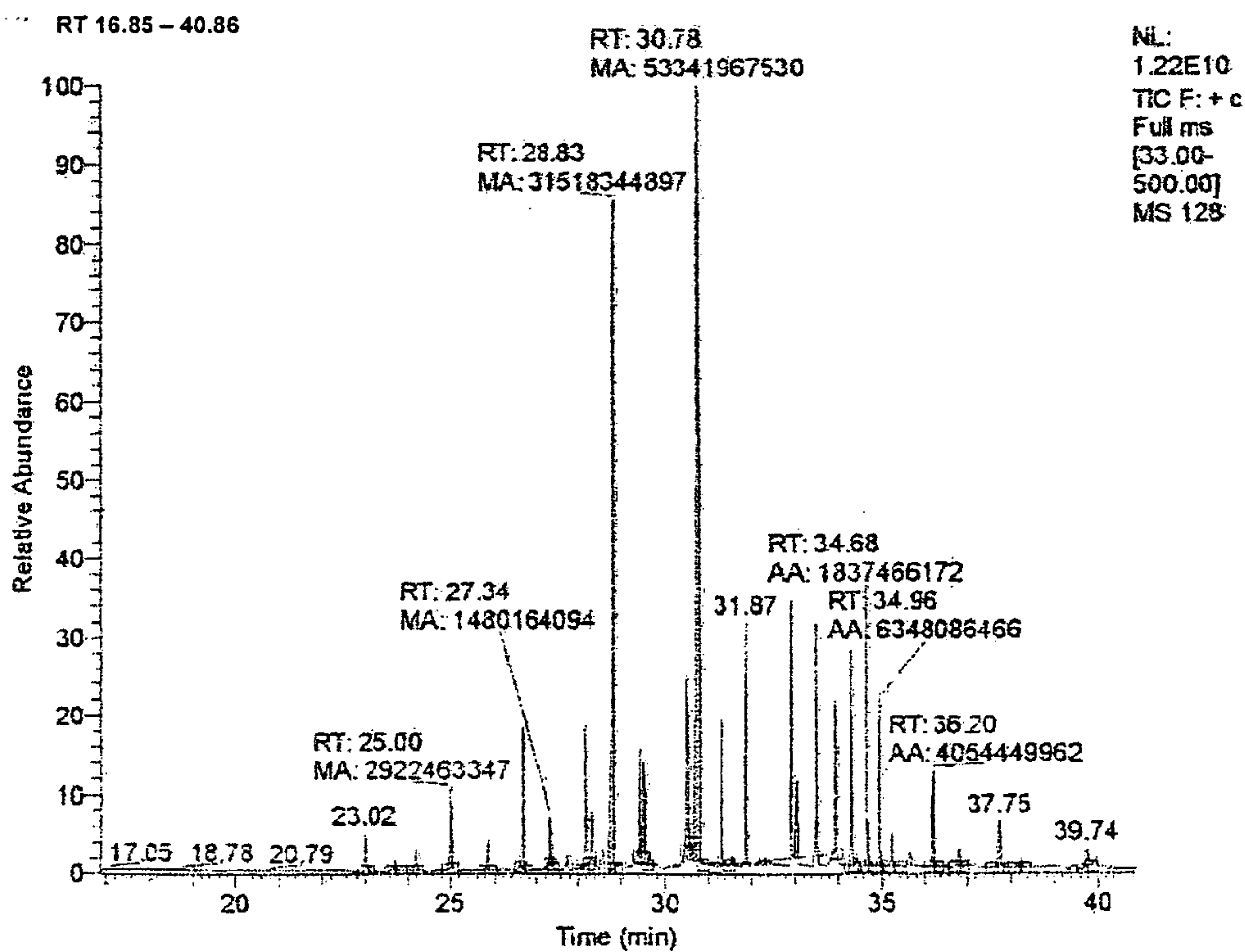


Figure 13

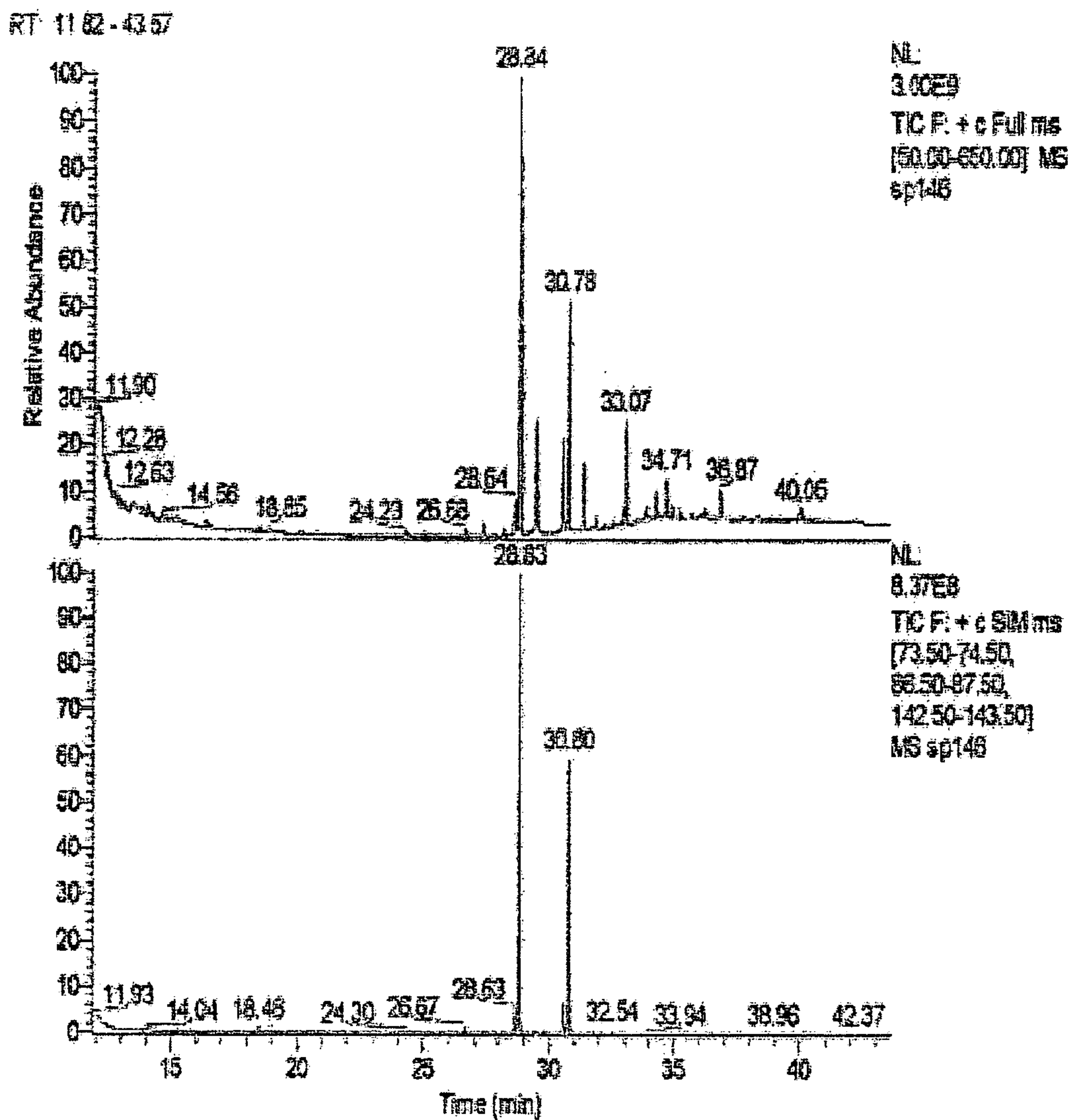


Figure 14

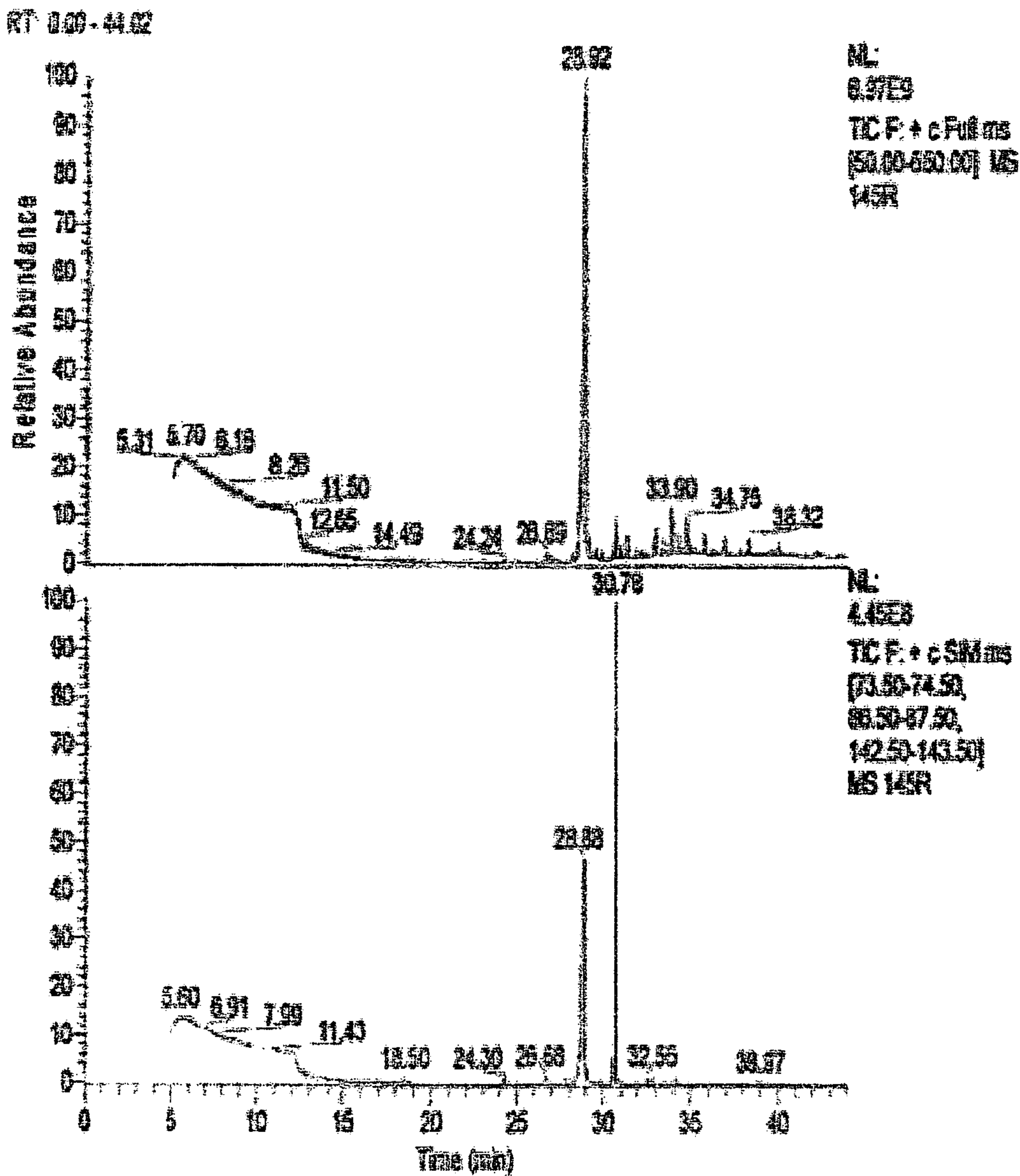


Figure 15

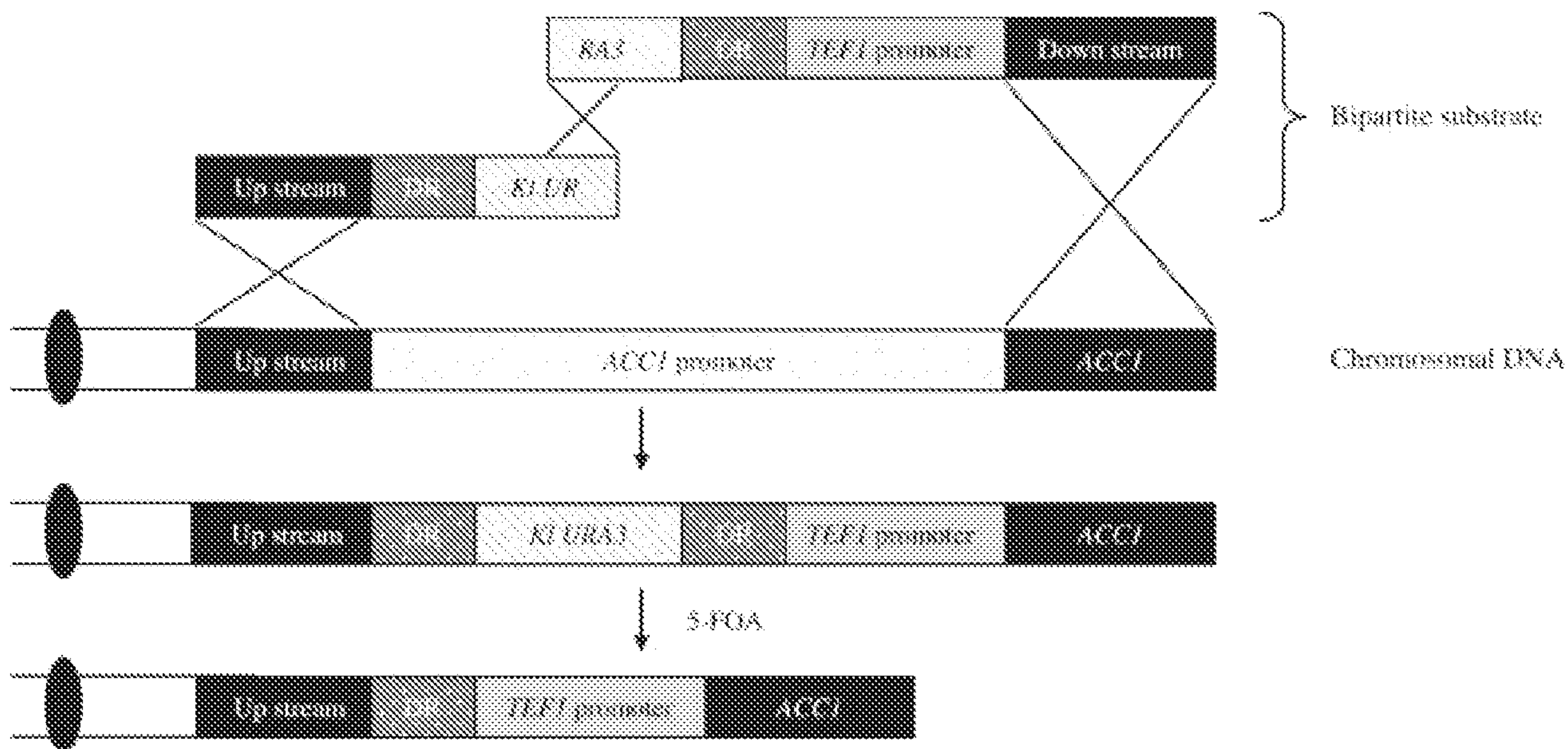
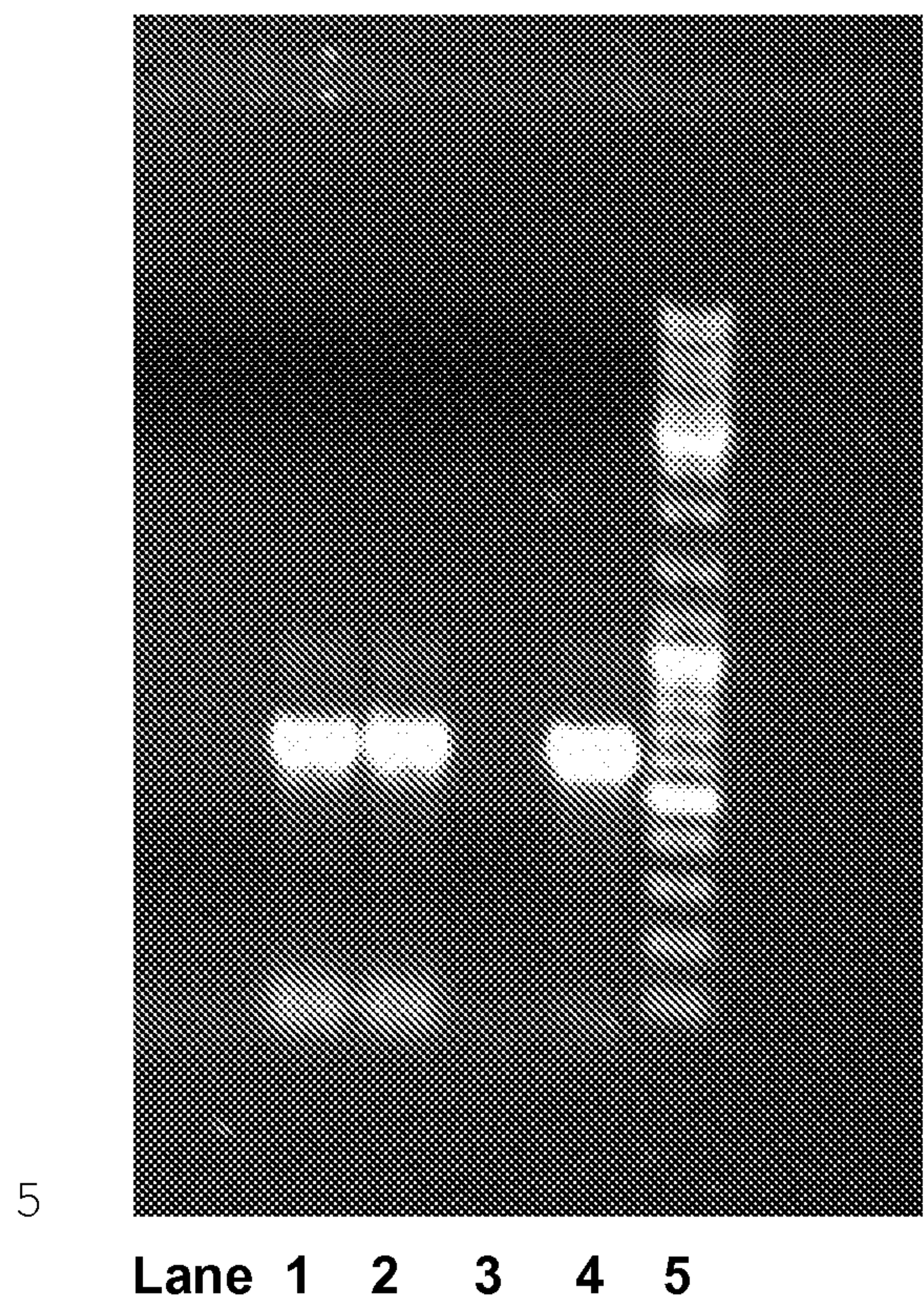
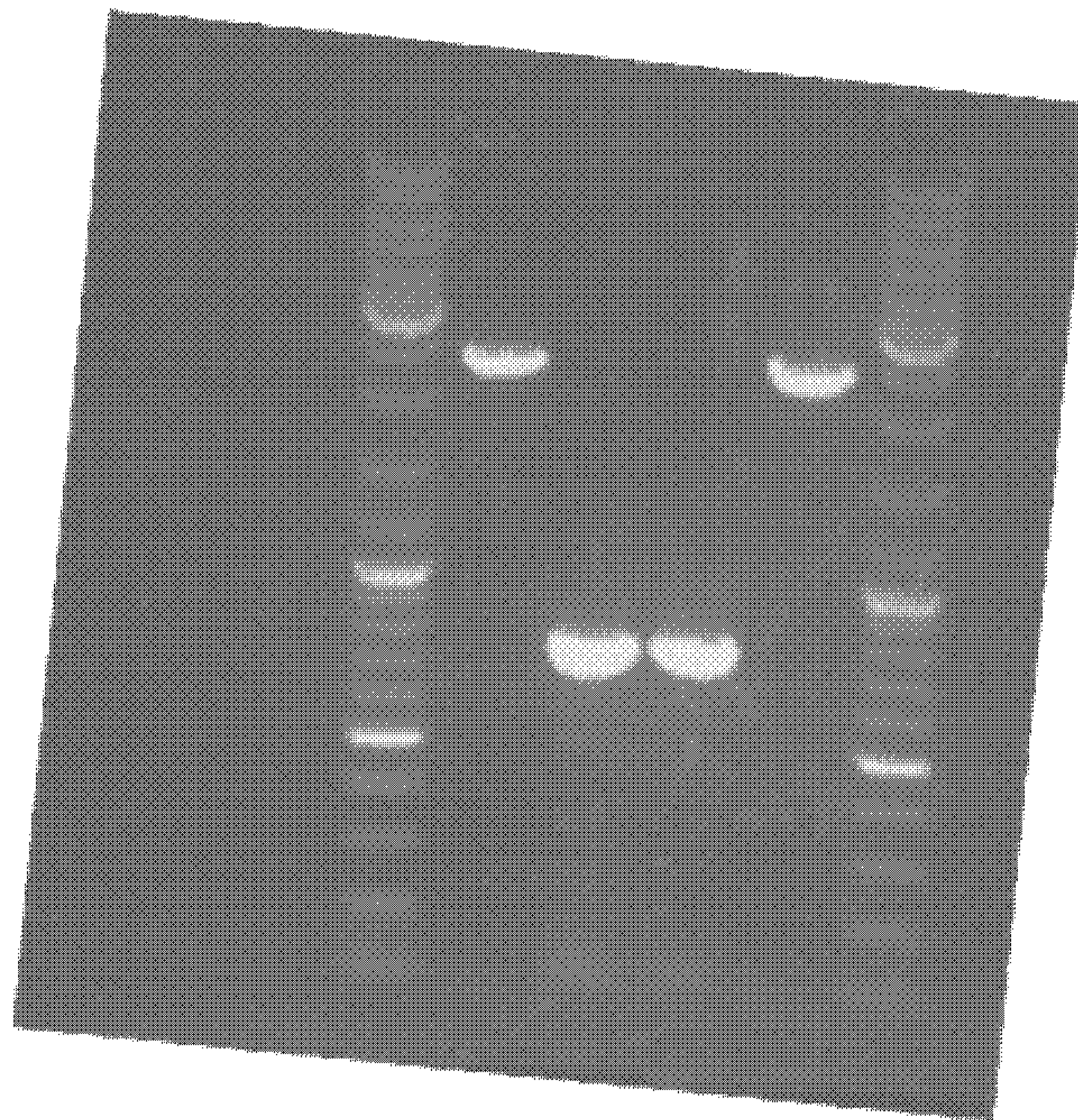


Figure 16



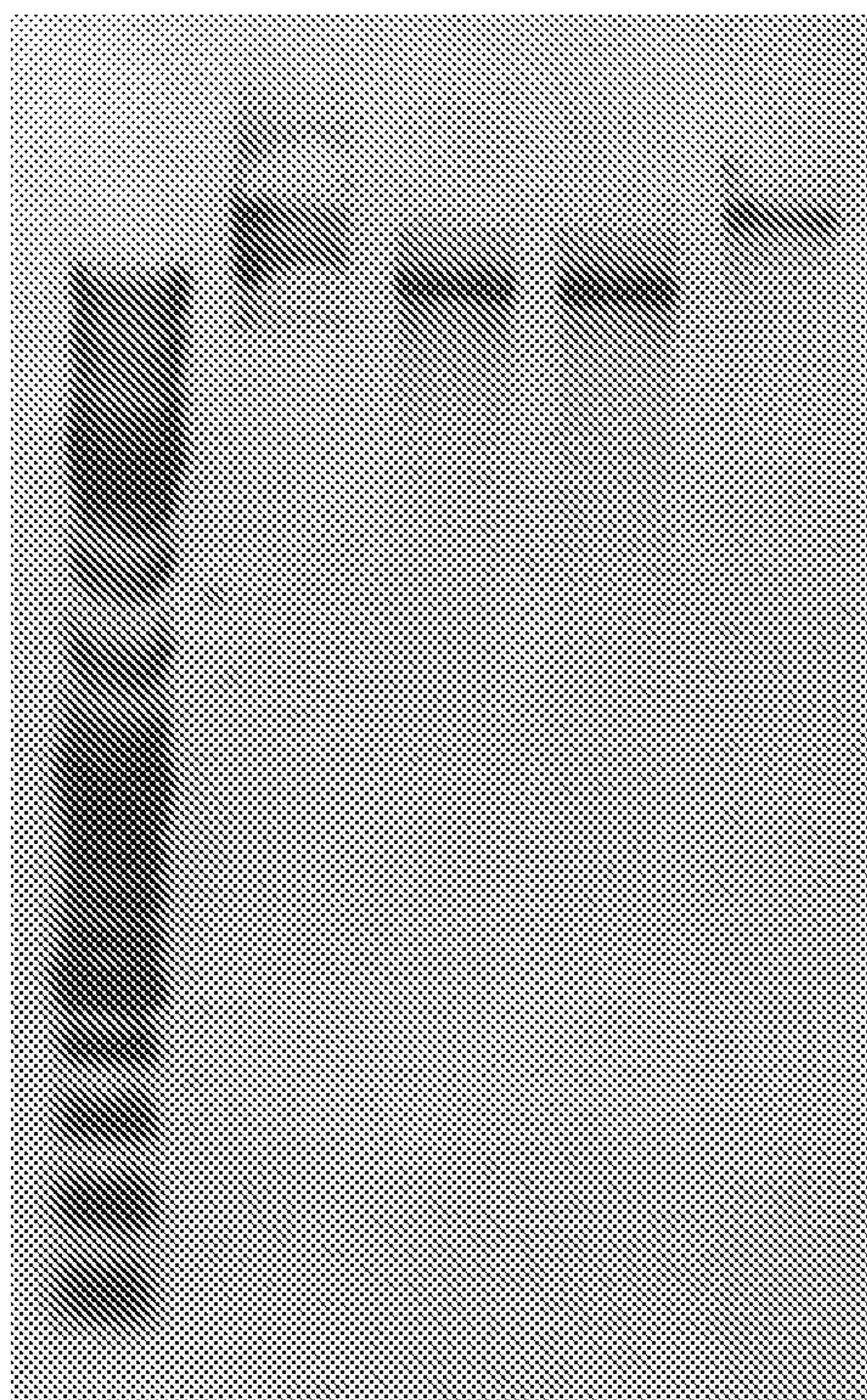
Lane 1 2 3 4 5

Figure 17



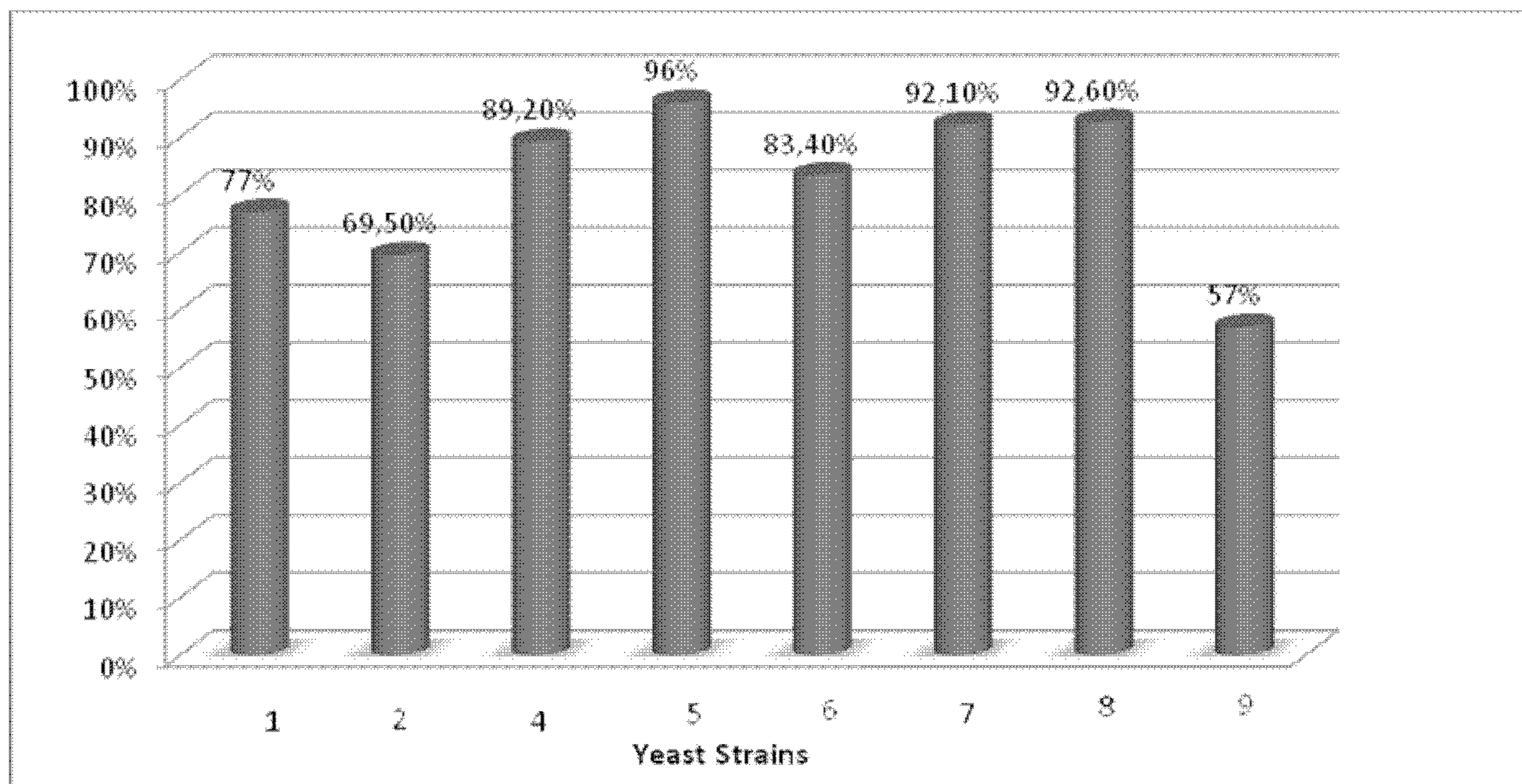
Lanes: M 1 2 3 4 M

5 **Figure 18**



M 1 2 3 4 Lane

Figure 19



5 **Figure 20**

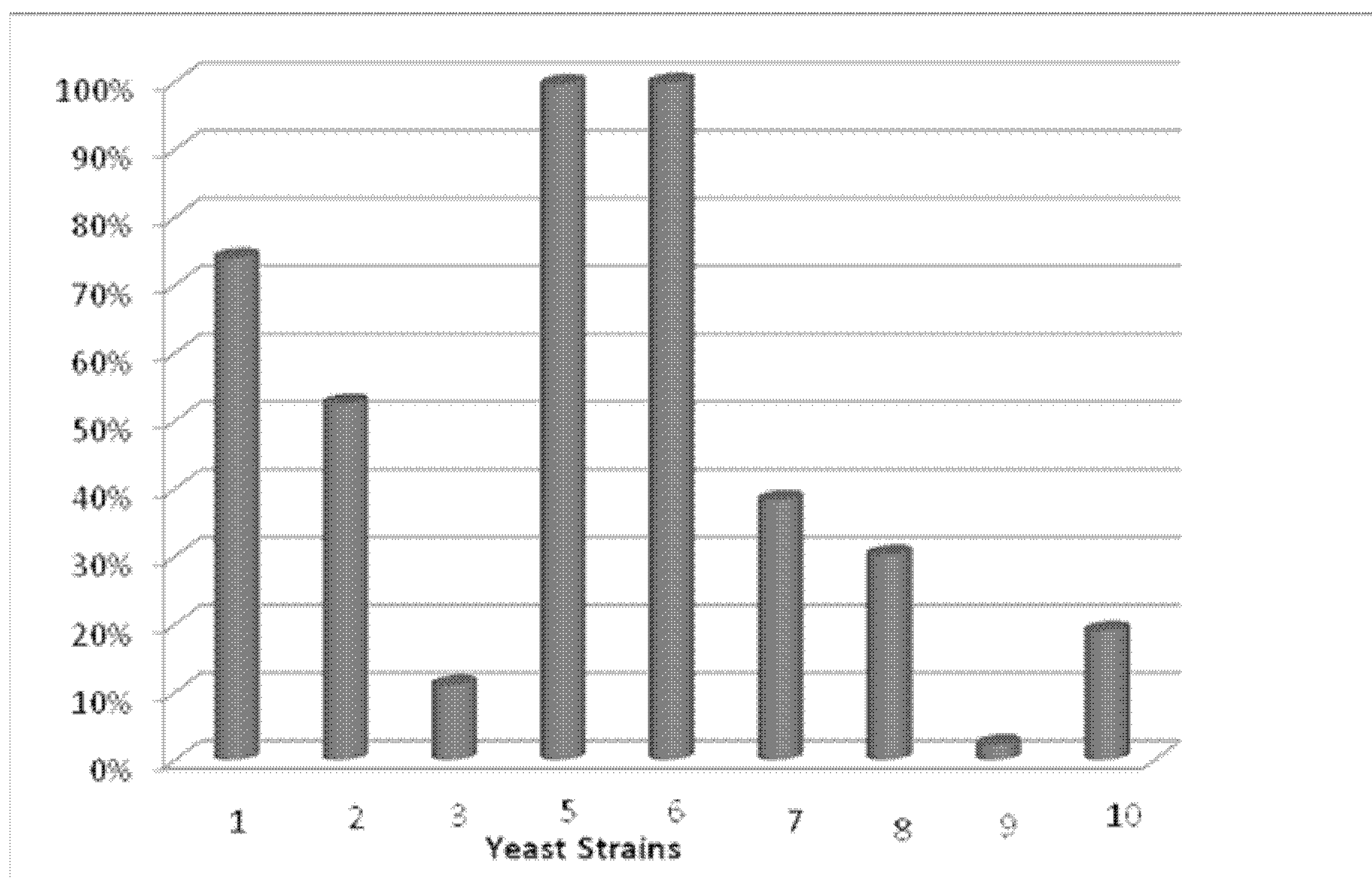


Figure 21

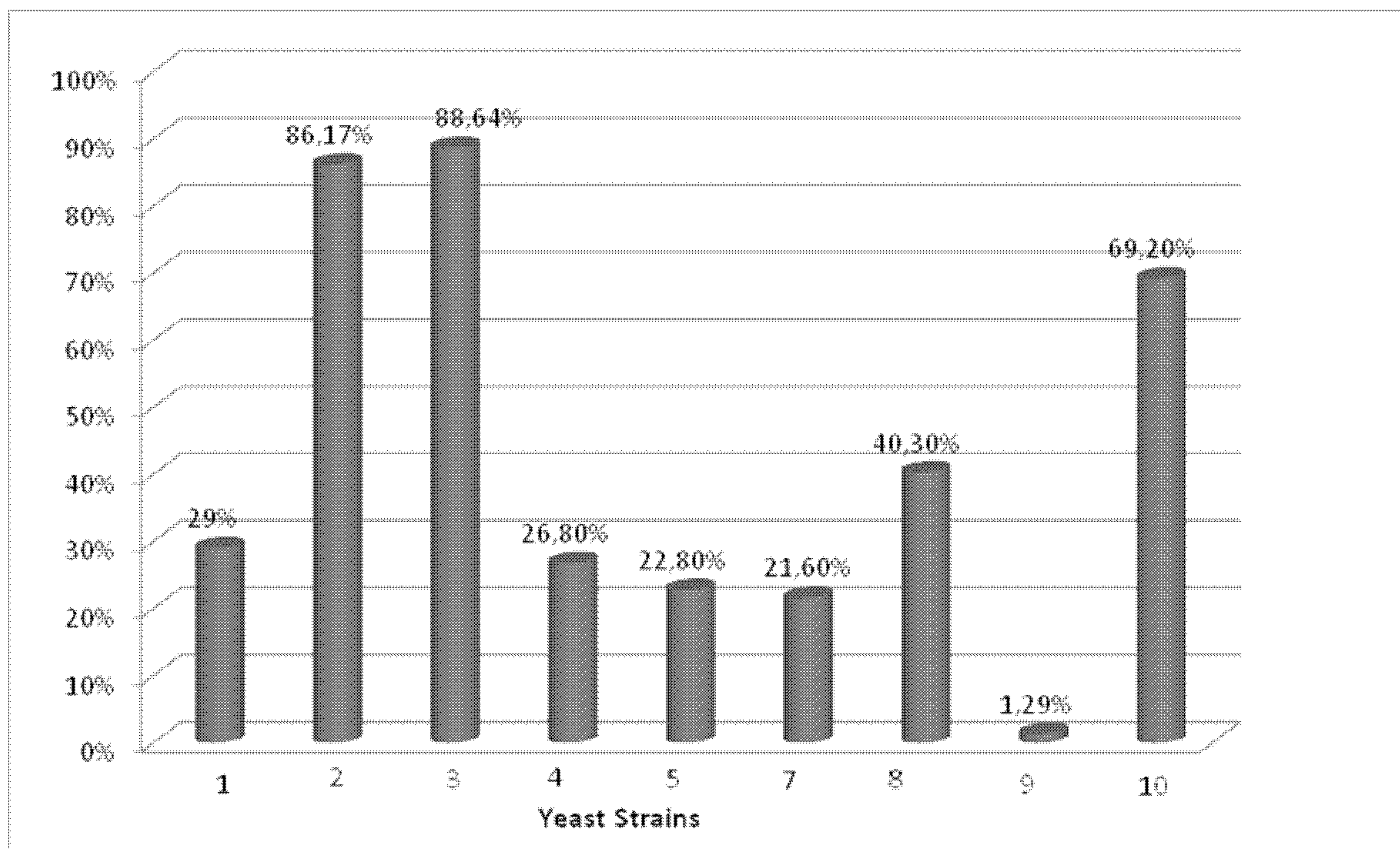


Figure 22

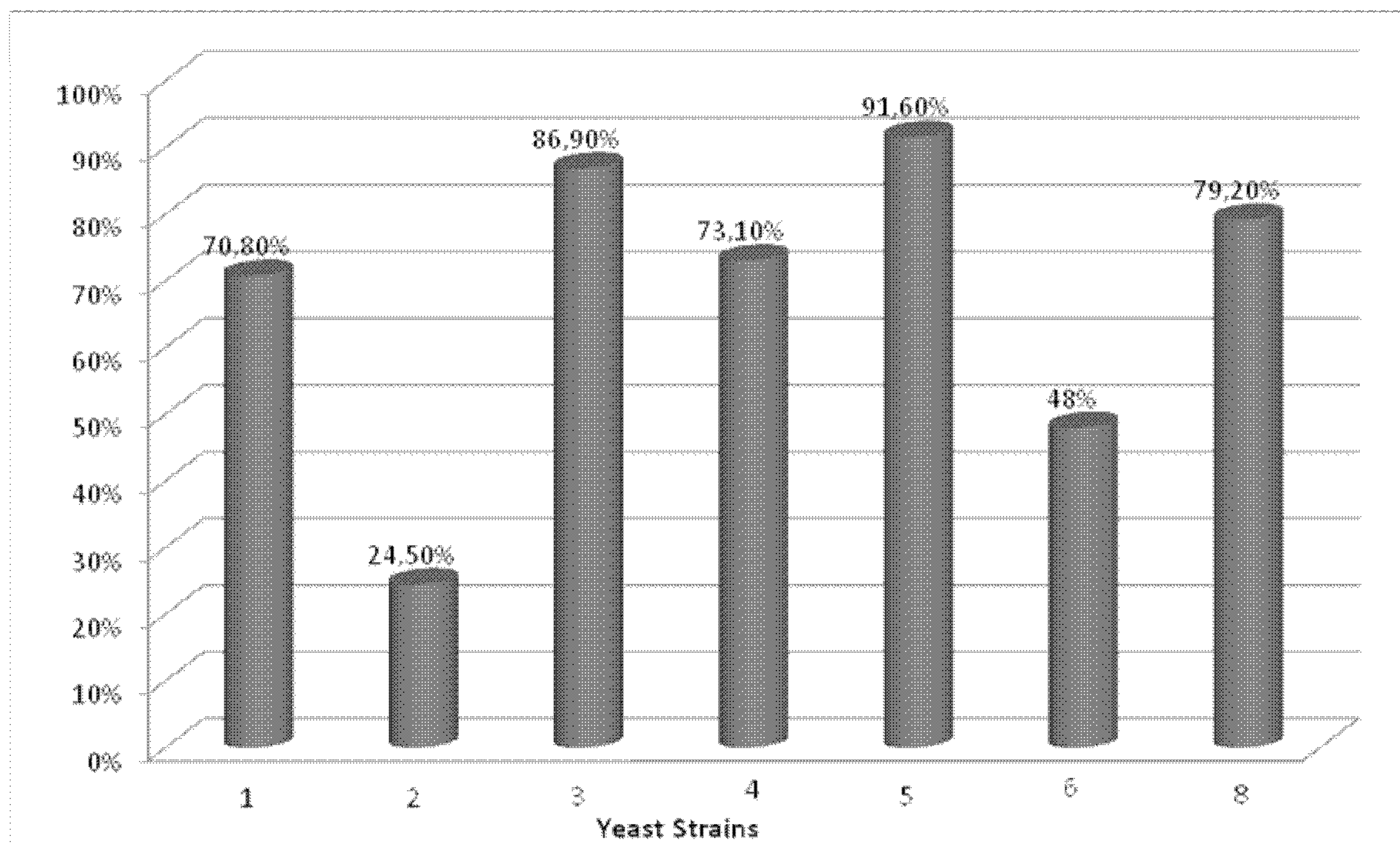


Figure 23

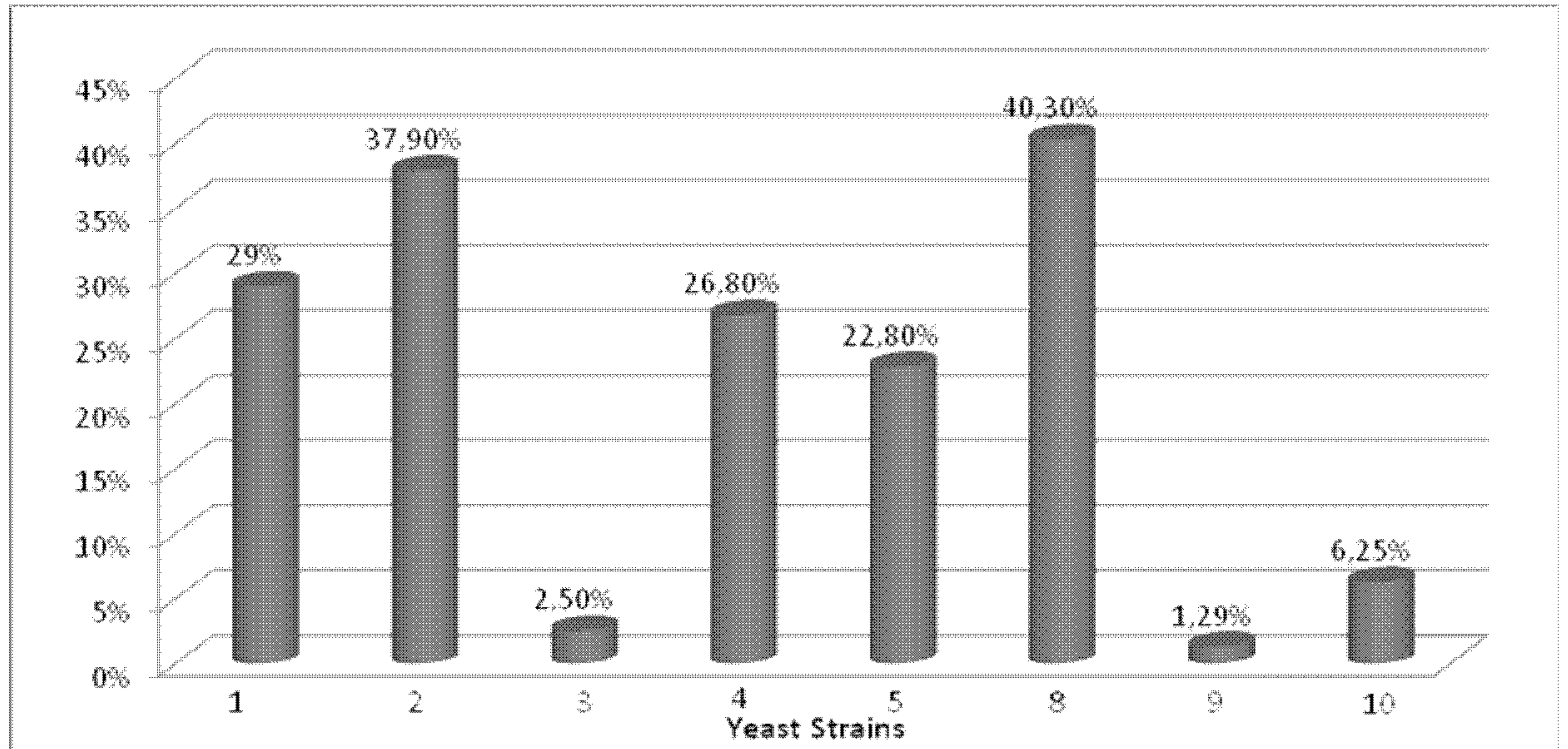


Figure 3b

