Zoetis Manufacturing Italia S.r.l.

Capitale Sociale Euro 29.000.000,00 interamente versato
P.IVA, C.F. e Iscr. al Registro delle Imprese di Catania: 05006160872
Iscrizione al R.E.A. di Catania n. 336207

Spett/le
Università di Palermo
Viale delle Scienze, Ed. 16,
90128 Palermo

Catania, 6 febbraio 2020
Prot. 62/2020/CT

Contratto di Servizi Prot. 204/2017/CT

Gentili Signori,
In riferimento al contratto di Servizi sottoscritto in data 01/06/2017 (di seguito il "Contratto") ed in particolare all'Exhibit A e, facendo seguito agli accordi intercorsi, in considerazione del fatto che per l'attività 2.3 Voi avete riscontrato delle difficoltà di natura esecutiva e che da parte nostra abbiamo valutato la non utilità della prosecuzione della stessa, mentre si ravvisa concordemente la necessità di una rimodulazione delle attività, dei costi e della relativa tempistica, si conviene e stabilisce quanto segue:

Rif 1. Current nemadectin production process improvement

1.1: Knockin and/or knockout of the genes involved in the biosynthesis of metabolite and/or its precursors.

Deliverable:	Selection of nemadectin high productivity *S. cyaneogriseus* strains
Acceptance Criteria:	25-30% higher yield/productivities; same impurities profile as current process
Duration:	approx. 3 months
Costs:	2,700 Euros

L'attività viene rimodulata, ampliata nei contenuti ed estesa fino a 21 mesi. Una prima parte iniziale è stata eseguita e saldata per 2,700 euro. L'attività è ancora in corso e si completerà il 31 Maggio 2020. Le parti concordano per la realizzazione di tutte le attività previste un costo complessivo di € 32.700,00, di cui:
€ 2,700 euro già corrisposte nel 2017 (vedi sopra);
€ 10.000,00 da corrispondere da parte di Zoetis per l'attività svolta nel 2019;
€ 20.000,00 si intende da corrispondere al completamento dell'attività e sulla base dei risultati (deliverables).

1.2 Use of scaffolds in fermentation for nemadectin production improvement.

Deliverable:	Set up of immobilised-cell fermentations for standard strain and for at least 1 selected mutant.
Acceptance Criteria:	25-30% higher yield/productivities; same impurities profile as current process
Duration:	approx. 6 months
Costs:	50,000 Euros

L'attività è stata svolta secondo i deliverables previsti ma non ha incontrato i criteri d'accettazione e pertanto si è deciso di discontinuarla per focalizzare gli sforzi sull'obiettivo

Rif 2. Metabolic engineering for 23-keto f-alpha production ("Green Process")

2.1 Knockin and/or knockout of genes involved in the biosynthesis of the 23-keto f-alpha metabolite and/or precursors.

Deliverable:	Selection of at least 1 clone stably transformed.
Acceptance	Criteria: markers maintained for > 30 passages
Duration:	approx. 13 months
Costs:	60,000 Euros

L'attività è stata svolta e pagata nel 2018 incontrando i deliverables ed i criteri d'accettazione previsti.

2.2: Mutation and/or overexpression of regulatory genes of the biosynthesis of the 23-keto f-alpha secondary metabolite.

Deliverable:	Selection of at least 1 clone stably transformed.
Acceptance Criteria:	produce baseline level of 23-keto f-alpha secondary metabolite with same impurities profile as of current nemadectin process.
Duration:	approx. 13 months
Costs:	50,000 Euros

L'attività è in corso e viene rimodulata ed estesa fino a 21 mesi (data di completamento: 31 Maggio 2020) per un importo complessivo di € 50.000 per le ragioni descritte nella relazione sull'attività del 2019 da parte di UNIPA. Le parti concordano un pagamento di:
€ 10.000,00 da corrispondere da parte di Zoetis per l'attività svolta nel 2019;
€ 40.000,00 si intende da corrispondere al completamento dell'attività.

2.3: Targeted amplification of the gene cluster for the synthesis of the 23-keto f-alpha secondary metabolite.

Deliverable:	Selection of at least 1 clone (markers maintained for > 30 passages)
Acceptance Criteria:	25-30% higher yield/productivities vs baseline level of 23-keto f-alpha secondary metabolite with same impurities profile as of current nemadectin process.
Duration:	approx. 6 months
Costs:	60,000 Euros

Le attività previste vengono cancellate e pertanto il presente punto viene escluso dallo scopo del lavoro

L'Exibit A viene pertanto sostituito dal presente

Exhibit "A"

1. Current nemadectin production process improvement

1.1: Knockin and/or knockout of the genes involved in the biosynthesis of nemadectin and/or its precursors.

Deliverable:	Selection of nemadectin high productivity *S. cyaneogriseus* strains
Acceptance Criteria:	25-30% higher yield/productivities; same impurities profile as current process
Duration:	approx. 36 months
Costs:	32.700,000 Euros

Activity description

a. Selection of a minimum of 3 target genes putatively involved in nemadectin production improvement on the base of scientific literature and/or bioinformatics approaches:
   i. bioinformatics analysis of nemadectin biosynthetic gene cluster of metabolic pathways leading to nemadectin biosynthesis to identify putative bottleneck affecting production;
   ii. study of scientific reports and papers describing strategies for production improvement of biologically active metabolites in actinomycetes;
   iii. design of strategies for nemadectin production improvement based on genetic manipulation.

b. Synthesis and/or PCR amplification of a minimum of 3 different DNA constructs containing selected genes:
   i. primer pair design for polymerase chain amplification of target gene(s) to be cloned into specific actinomycete expression vector that will be chosen on the base of scientific literature;
   ii. depending on size of target gene(s) and the desired characteristics of final construct, polynucleotide macromolecules will be synthetized on the base of open reading frame (ORF) and regulatory region sequences needed to obtain the final construct to be cloned into specific actinomycete expression vector chosen as above described.

c. Construction of a minimum of 3 different recombinant plasmids to obtain *S. cyaneogriseus* recombinant strains:
   i. validation of nucleotide identity by restriction digestion analysis and/or sequencing and bioinformatics analysis of constructs to be cloned into expression vector;
   ii. digestion and purification of expression vector and insert;
   iii. performing ligation protocol to obtain recombinant plasmid using digested vector and insert;
   iv. transformation of competent *Escherichia coli* cells and cultivation and isolation on selective growth medium;
   v. plasmid recovery and electrophoretic analysis of restriction digestion pattern and validation of construct identity by PCR analysis coupled with PCR amplicon purification, sequencing and bioinformatics analysis;

d. Isolation of a minimum of 3 *S. cyaneogriseus* recombinant strains for each recombinant plasmid obtained for nemadectin production improvement
   i. transformation of *E. coli* cells suitable for transferring foreign DNA into actinomycetes;
   ii. transformation and isolation by antibiotic resistance selection of a minimum of 3 *S. cyaneogriseus* recombinant strains for each recombinant plasmid obtained;
   iii. molecular characterization of isolated strains by PCR amplification, PCR amplicon purification and sequencing and bioinformatics analysis to confirm the presence of recombinant plasmid;

e. Delivery of a minimum of 3 recombinant strains per each recombinant plasmid to Zoetis .:
   i. the confirmed *S. cyaneogriseus* recombinant strains will be tested for stably maintenance of selection marker up to 30 passages;
   ii. stable *S. cyaneogriseus* recombinant strains will be delivered to Zoetis for production test.

1.2 Use of scaffolds in fermentation for nemadectin production improvement.

Deliverable:	Set up of immobilised-cell fermentations for standard strain and for at least 1 selected mutant.
Acceptance Criteria:	25-30% higher yield/productivities; same impurities profile as current process
Duration:	approx. 6 months
Costs:	50.000 Euros

Activity description

Activity completed

2: Metabolic engineering for 23-keto f-alpha production ("Green Process")

2.1: Knockin and/or knockout of genes involved in the biosynthesis of the 23-keto f-alpha metabolite and/or precursors.

Deliverable:	Selection of at least 1 clone stably transformed.
Acceptance	Criteria: markers maintained for > 30 passages
Duration:	approx. 13 months
Costs:	60,000 Euros

Activity description

Activity completed

2.2: Mutation and/or overexpression of regulatory genes of the biosynthesis of the 23-keto f-alpha secondary metabolite.

Deliverable:	Selection of at least 1 clone stably transformed.
Acceptance Criteria:	produce baseline level of 23-keto f-alpha secondary metabolite with same impurities profile as of current nemadectin process.
Duration:	approx. 21 months
Costs:	50.000 Euros

Activity description

a. Due to the absence of information concerning the regulatory processes and metabolic pathways that can be related to the biosynthesis of the 23-keto f-alpha secondary metabolite in *S. cyaneogriseus*, a proteome analysis will be carried out to unveil proteins and genes thereof that can be associated with secondary metabolite biosynthesis:

i. setting up of an experimental design aimed at revealing proteins and genes thereof up- or down-regulated during secondary metabolite production in *S. cyaneogriseus* fermentations;
ii. identification by mass spectrometry analysis of the proteins up- or down-regulated during secondary metabolite production;
iii. bioinformatic reconstruction of metabolic pathways and regulatory processes positively or negatively associated with secondary metabolite production.
iv. list of target genes to obtain *S. cyaneogriseus* recombinant strains with improved f-alpha and/or 23-keto f alpha.

b. design of genetic strategies based on mutation of C23 ketoreductase domain (KRD-C23) of polyketide synthase (PKS) module involved in the ketoreduction of C23 hydroxy group;

i. bioinformatics analysis of nemadectin biosynthetic gene cluster aimed at revealing ketoreductase domains of PKS module putatively involved in the reduction of C23 hydroxy group;
ii. design of a minimum of 2 DNA constructs carrying in frame deletion mutations in KRD-C23;
iii. synthesis of DNA constructs containing mutated KRD-C23 for cloning into pCRISPR-CAS9 system vector.

c. preparation of pCRISPR-CAS9 system vectors for generation of *S. cyaneogriseus* strains having mutated KRD-C23:

i. synthesis of a minimum of 2 oligonucleotides complementary to KRD-C23 encoding sequence to generate sgRNA cassettes for targeting CAS9 cleavage specifically;
ii. cloning of the specific sgRNA cassette into pCRISPR-CAS9 vector and validation of construct identity by PCR analysis coupled with PCR amplicon purification, sequencing and bioinformatics analysis;
iii. cloning DNA into pCRISPR-CAS9/sgRNA vector of construct encoding mutated KRD-C23 and validation of construct identity by PCR analysis coupled with PCR amplicon purification, sequencing and bioinformatics analysis;
iv. transformation and isolation by antibiotic resistance selection of a minimum of 3 *S. cyaneogriseus* recombinant strains for each DNA construct;
v. induction of CRISPR-CAS9/sgRNA-based homologous recombination to obtain deletion mutants for KRD-C23 encoding domain;
vi. molecular characterization by PCR analysis coupled with PCR amplicon purification, sequencing and bioinformatics analysis to confirm deletion of KRD-C23 domain encoding sequence in *S. cyaneogriseus*.

d. delivery to Zoetis S.r.l. of a minimum of 3 mutated strains per each construct stably transformed for 23-keto f-alpha production assays:

i. stable *S. cyaneogriseus* recombinant strains will be delivered to Zoetis S.r.l for production test.

2.3: Deleta

Distinti saluti

Zoetis Manufacturing Italia s.r.l.

Per accettazione.

UNIPA

Dipartimento STEBICEF
Il Direttore
Prof. Silvestre Buscemi

Firmato digitalmente da:Silvestre Buscemi
Organizzazione:UNIVERSITA' DEGLI STUDI DI PALERMO/80023730825
Data:26/03/2020 13:37:20