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Date: 09-June-2020

To **Final Due Date: 10-June-2020**

The Controller of Patents,
The Patent Office,
Plot No. 32, Sector 14, Dwarka
New Delhi -110075.

Kind Attention: Dr. Sunil Kumar, Controller of Patents

Subject: Reply of First Examination Report

Application No: **0984DEL2014**
Filing Date**: 04-April-2014**
FER Date**: 10-Dec-2019**
IPU Ref. No: **0068NF2014**

Dear Madam,

With reference to the First Examination Report issued in respect of the subject patent application vide your reference no -0984/DEL/2014 dated 10/12/2019, the applicant hereby submits the under mentioned documents with the following observations for the objection in examination report.

OBSERVATIONS

Regarding Part II (B)/ NOVELTY

Regarding the objection contained in this paragraph, it is submitted that the subject matter of the claimed invention is Novel as per the section 2 (1)(j) of The Indian Patent Act, 1970 , in view of the cited prior art document for the following reasons **:**

D1: DATABASE UniProt [Online],16 August 2004 (2004-08-16). SubName: Full= Putative exported choloylglycine hydrolase UNIPROT:Q6D291,Database accession no. Q6D291 sequence The Uniprot entry Q60291 shown in D1 (Refseq: WP _011 094726, formerly YP _051294.1) discloses a sequence comprising the entire SEQ ID NO 1, the only difference being the database sequence comprises the N-terminal periplasmic signal peptide (amino acids 1-29). Claim 1 is directed to the enzyme consisting of the sequence set forth in SEQ ID NO 1.

Our Submission- D1 does not provide any teaching with respect to the nucleotide sequence (Seq Id No.2) encoding the penicillin acylase enzyme having Seq Id No.1. Moreover, the process relating to recombinant DNA technology for the expression of penicillin V acylase in the cytoplasmic soluble region of the host cell is not disclosed in D1. The expressed protein of the present invention has a methionine residue added before the N-terminal cysteine, which is later processed by simple removal of methionine in the host E. coli. The protein is expressed in *E. coli* with a C-terminal 6X His-tag, in the cytoplasmic soluble fraction.

Therefore, in view of the currently amended claim 1 and the fact that D1 does not teach recombinant expression of PVA enzyme, the present invention is novel over D1.

D2:Avinash VS, Panigrahi P , Suresh CG, Pundle AV , Ramasamy S. Structural modelling of substrate binding and inhibition in penicillin V acylase from Pectobacterium atrosepticum. Biochemical and biophysical research communications. 2013 Aug 9;437(4):538-43.

D2 shows purification of the P. atrosepticum penicillin V acylase, whereby an activity of 16.612 IU/mg was obtained (Table 1). Homology modelling based on the amino acid sequence YP _051294.1 (which is the same as the one disclosed in D1) is shown. For the modelling, the N-terminal 29 residue signal peptide was removed (p. 539, left column, last paragraph). Hence, D2, in fact, discloses the amino acid sequence of SEQ ID NO 1. Since the sequences are identical, the physicochemical properties now incorporated into claim 1, i.e. the stability at pH 3-6 and temperature from 20-50 °C are inherently present in the molecule disclosed in D2

Our Submission – D2 does not disclose the nucleotide sequence represented by Seq Id No. 1. D2 focusses on the structural homology, physiological properties and the enzyme docking

studies of PVA. There is no teaching in D2 relating to the recombinant expression of the penicillin V acylase in a host cell and its expression in the cytoplasmic region of the host cell.

On the other hand, the expressed protein in the present invention has a methionine residue added before the N-terminal cysteine, which is later processed by simple removal of methionine in the host E. coli. The protein is expressed in *E. coli* with a C-terminal 6X His-tag, in the cytoplasmic soluble fraction. D2 does not provide any such expression of the protein in the cytoplasmic region.

The specific activity of the enzyme of D2 is estimated to be 16.612 IU/mg (Refer Table 1 of page no. 539 of D2). The recombinant PVA enzyme synthesized in the present invention as claimed in amended claim 1 has a specific activity of 434 IU/g and which when stored at 4°C is stable and retains activity after 30 days.

Further, the enzyme expressed in the cytoplasmic region of the host cell is immobilized on a suitable matrix to improve its stability and reusability. The enzyme synthesized by the instant process may be immobilized on solid support such as alginate, polyvinyl alcohol, acrylamide, Eupergit C and such like to improve the stability and reusability for industrial applications.

Therefore, the subject matter of amended claims 1 and 2 of the present invention are novel over D2.
Therefore, the subject-matter of claims 1-3 can be acknowledged with novelty as required u/S. 2(1)(j) of the Patents Act, 1970 in view of the disclosure in the prior art documents D1-D2 as the subject matter of claims 1-3 are not anticipated in view of D1-D2.

Regarding Part II (B)/ INVENTIVE STEP

Regarding the objection contained in this paragraph , it is submitted that the subject matter of the claimed invention is inventive as per the section 2 (1)(ja) of The Indian Patent Act, 1970 , in view of the cited prior art documents for the following reasons :

D1: DATABASE UniProt [Online],16 August 2004 (2004-08-16). SubName: Full= Putative exported choloylglycine hydrolase UNIPROT:Q6D291,Database accession no. Q6D291 sequence

The Uniprot entry Q60291 shown in D1 (Refseq: WP _011 094726, formerly YP _051294.1) discloses a sequence comprising the entire SEQ ID NO 1, the only difference being the database sequence comprises the N-terminal periplasmic signal peptide (amino acids 1-29). Claim 1 is directed to the enzyme consisting of the sequence set forth in SEQ ID NO 1.

D2:Avinash VS, Panigrahi P , Suresh CG, Pundle AV , Ramasamy S. Structural modelling of substrate binding and inhibition in penicillin V acylase from Pectobacterium atrosepticum. Biochemical and biophysical research communications. 2013 Aug 9;437(4):538-43.

D2 shows purification of the P . atrosepticum penicillin V acylase , whereby an activity of 16.612 IU/mg was obtained (Table 1). Homology modelling based on the amino acid sequence YP _051294.1 (which is the same as the one disclosed in D1) is shown. For the modelling, the N-terminal 29 residue signal peptide was removed (p. 539, left column, last paragraph). Hence, D2, in fact, discloses the amino acid sequence of SEQ ID NO 1. Since the sequences are identical, the physicochemical properties now incorporated into claim 1, i.e. the stability at pH 3-6 and temperature fro 20-50 °C are inherently present in the molecule disclosed in D2.

Our Submission –

With respect to the inventive step of the recombinant penicillin V acylase as claimed in amended claim 1, one needs to observe that the said protein has been expressed in the cytoplasmic fraction of the host cell, therefore, the process steps of purification are considerably reduced. The process steps as described in the Example 3, page 11 of the present complete specification involves, cell lysis, centrifugation and Anionic exchange chromatography. On the other hand, D2 employs an additional step of ammonium sulphate precipitation.

Therefore, the costs involved in synthesis of the PVA as claimed in claim 1 is due to the cytoplasmic expression in the host cell. D1 and D2 do not provide any nucleotide or cDNA sequence for the synthesis of the PVA in a host cell.

The presently synthesized enzyme shows stability with no significant changes in secondary structure till 70°C (Fig. 4 of the present invention).

Moreover, the specific activity of the presently synthesized enzyme is approximated 30 times greater that the enzyme of D2. The specific activity of the enzyme of D2 obtained by cultivation of *P. atrosepticum* DSM 30186 is estimated to be 16.612 IU/mg whereas the presently claimed PVA in amended claim 1 has a specific activity of 434 IU/g and which when stored at 4°C is stable and retains activity after 30 days.

Further, the present invention provides recombinant PaPVA yielding 60 mg/ml protein from 1 g recombinant cells, thus amounting to 250-300 mg protein/L culture. However, D2 in table 1 indicates a yield of protein of 1mg/2.4 ml from a 10% inoculum of P. atrosepticum DSM 30186.

Therefore, it is evident that the present invention has tremendous technical skill and economic significance over the enzyme of D2.

As regards inventive step of amended and renumbered claim 6 relating to immobilization and the amended claim 8 reciting the present recombinant bacterial strain of the present enzyme the attention of the controller needs to be brought to the table at example 8, page 16 of the present complete specification. The present inventors have demonstrated the enhanced free cell activity and cell bound activity and activity recovery of the present enzyme.

It is submitted that neither D1 nor D2 provide any teaching relating to the following technical features of the present invention:

- **(i) Recombinant expression of PVA with enhanced specific activity**
- **(ii) Expression of PVA in the cytoplasmic region of the host cell as a soluble fraction, thereby mitigating costs involved in enzyme purification**
- **(iii) High yield of the purified enzyme**

(iv) Recombinant host cell having deposition no. of MCC0018 exhibits high substrate specificity of 434 IU/g, which is 30 fold greater than that of the enzyme secreted by the native strain of of *P. atrosepticum* DSM 30186.

D3: US 2005142652 (A1), 30 June 2005. D3 relates to a recombinant plasmid of FIG. 1, wherein (1) is pET-26b(+) cloning/expression region with SEQ ID No. 1 cloned between BamH I site 198 and Nde I site 288, (2) is lac I coding sequence, (3) is pBR322 origin, (4) is Kan coding sequence, and (5) is f1 origin; a recombinant E. Coli strain PTA 2456; and lastly, a process for the production of large amount of Penicillin V acylase using recombinant E. Coli strain PTA 2456.

The inventiveness for the claims 1- 3 cannot be ascertained as these do not have any novel feature u/s 2(1) (j) of the Indian Patents Act. The closest prior art to the subject-matter of the present set of claims is D2.D2 concerned with purification and characterization of the P . atrosepticum penicillin V acylase of SEQ ID NO 1. The difference of the subject-matter in claims 4-10 are the vectors, host cells, the immobilized enzyme, the process for preparation of recombinant enzyme and a recombinant bacterial strain carrying the gene comprising SEQ ID NO 1. It appears that these differences all relate to trivial embodiments that are within the routine practice of a person skilled in the art once the purified enzyme and its sequence is at hand (as is the case in D1). The techniques are illustrated in D3, where vectors, host cells, recombinant expression etc. of a penicillin V acylase from B. subtilis is shown (e.g. claims 1, 3, 6). Therefore, it appears that the subject-matter of claims 3-10 lacks inventive step over D2 and the general knowledge of a person skilled in the art or over D2 in combination with D3.

<u>Our Submission</u> – As regards D3, the disclosure does not provide any teaching suggestion or motivation to a person skilled in the art for cytoplasmic expression of the PVA from Bacillus subtilis. Moreover, the organism employed in PVA expression in D3 is different from that of the present invention. Therefore, the recombinant DNA tools required for protein expression in D3 are different from that of the present invention.

Even though PVA synthesis has been demonstrated in D3, there is no ease and convenience of purification of the end product due to the secretion of the enzyme in the periplasmic region.

D3 teaches expression in Bacillus spp. Which is a gram-positive organism. *Pa*PVA of the present invention has only 25% sequence similarity with already characterized PVAs from Bacillus and BSHs from Gram-positive bacteria, which furthers the argument for the uniqueness of the enzyme discussed in the present invention.

The final yield of protein was 62 mg/ g wet cells, with a specific activity of 434 IU/mg. PaPVA exhibits many folds greater specific activity for Pen V than other PVAs from Gram-positive bacteria, actinomycetes, fungi and yeast. Such high activity and high protein yields make PaPVA a valuable enzyme for use in the pharmaceutical industry.

Neither D1, D2 nor D3, either alone or combined, fall under presently claimed subject matter.

Accordingly, in view of the aforesaid, the applicant respectfully requests the reconsiderations of the amended claims 1-10 under examination and submit that the claims do not fall within the purview of section 2(1) (j a) of the Indian Patents Act and therefore may be allowable. The learned controller is therefore requested that the objection in this regard may be withdrawn.

Regarding Part II (B)/ NON-PATENTABILITY:

1. Claims 1-7 fall within the scope of clause (d) of section 3 of the Patents Act 1970 (as amended) as the preamble of the said claims relate to a recombinant penicillin V acylase enzyme, which is directed towards the new use/new form of a known substance. Therefore, the said claims are not allowable u/s 3(d) of the Patents Act 1970 (as amended).

2..Claims 8-10 fall within the scope of clause(d) of section 3 of the Patents Act 1970 (as amended) as it relates to process for the preparation of the recombinant penicillin V acylase.As the process has already been disclosed in the prior art D1-D3,which directed towards the mere use of known process . Therefore, the said claims are not allowable u/s 3(d) of the Patents Act 1970 (as amended).

Our Submission – As regards the objection raised in this paragraph, it may kindly be noted that the specific activity of the presently synthesized enzyme is approximated 30 times greater that the enzyme of D2. The specific activity of the enzyme of D2 obtained by

cultivation of *P. atrosepticum* DSM 30186 is estimated to be 16.612 IU/mg whereas the presently claimed PVA in amended claim 1 has a specific activity of 434 IU/g and which when stored at 4°C is stable and retains activity after 30 days.

Further, one needs to observe that the said protein has been expressed in the cytoplasmic fraction of the host cell, therefore, the process steps of purification are considerably reduced. The process steps as described in the Example 3, page 11 of the present complete specification involves, cell lysis, centrifugation and Anionic exchange chromatography. On the other hand, D2 employs an additional step of ammonium sulphate precipitation, therefore incurring increased costs.

In view of the recombinant expressed PVA enzyme claimed in amended claim 1 having an enhanced specific activity and the high yield of protein obtained by the present process compared to the PVA of D2, the presently amended claims do not come under the purview of Section 3(d) of The Indian Patent Act,1970. Withdrawal of objection in this regard is therefore humbly requested.

Regarding Part II (B)/SUFFICIENCY OF DISCLOSURE:

Details of depositing the biological material to an International Depository Authority under the Budapest Treaty: Biological materials information like Deposit in authorized depository institution, date of deposit, date/number of deposit in the specification and source/geographical origin in the specification shall be included in the body of complete specification.

Our Submission – As regards the objection contain in the above paragraph , it may kindly be noted that the details of depositing the biological material and source / geographical origin of biological material is given in the complete specification on page no-6, Detail Description of the invention , last paragraph. Therefore, it is humbly requested to waives the objection in this regard.

Regarding Part II (B)/CLARITY AND CONCISENESS:

Claims are indefinite, broad, vague and do not define the scope of the invention. Therefore, the claims do not comply with the requirements of section 10(4) (c) of The Patents Act 1970 (as amended).

Our Submission – In order to meet the objections raised in these paragraphs, the claims have been thoroughly amended.Therefore, it is humbly requested to waives the objection in this regard.

Regarding Part II (B)/ DEFINITIVENESS:

(A) Principal claim should be definitive and must contain all major technical features of the invention. Principal claim must clearly define the overall boundary of the claimed invention followed by dependent claims to give elaborate explanation. Claims must clearly define the scope of invention as per Sec 10[4(c)] of The Patents Act 1970.

(B) The details of the product should be given in claim 10.

Our Submission – In order to meet the objections raised in these paragraphs, the claims have been thoroughly amended

Regarding Part II OTHER REQUIREMENTS:

A. Amendments to the description/claims (if any) shall be submitted along with a marked-up copy clearly highlighting the corrections/amendments made in originally filed specification/claims (only the page/s in which amendment is proposed). Further, the support for the subject matter added to the claims should be clearly pointed out in the complete specification by mentioning the corresponding page(s) and paragraph(s) number(s).

B. Title of the complete specification shall be amended in conformity with the claim amendments so as to make it consistent with the amended claims if any.

C. Details regarding application for Patents which may be filed outside India from time to time for the same or substantially the same invention should be furnished within six months from the date of filing of the said application under clause (b) of subsection (1) of section 8 of Patents Act and

rule 12(1) of Patents Rules. If the prescribed time period under rule 12(1) has not been complied regularly, a fresh Form-3 shall be filed along with a petition to condone the irregularity.

Our Submission – A. As regards the objection raised in the above paragraph, it may kindly be noted that we have not added any subject matter or have not done any amendments in the complete specification.

B. Title of the complete specification is corrected which had been filed, we have not amended the title of the complete specification.

C. As regards the objection contained in this paragraph, it may be noted that we had timely filed Form 3.

REGARDING PART III (B) FORMAL REQUIREMENTS

Statement & Undertaking (Form 3 Details) – As regards the objection contained in this paragraph, it may be noted that we had timely filed Form 3.

Sequence listing is being re-submitted.

All the remaining requirements have been complied with.

Under the above, grant of the application within the final period is respectfully requested. In case the requirements are still not met, a hearing under section 14 of the Patents (Amendments) Act, 2005 is desired before the application is refused / rejected or treated as abandoned.

Yours Sincerely,

Dr. Indrani Ghosh

Enclosures:

- Amended claims – Mark-up Copy.
- Amended claims – Clear Copy.
- Sequence listing

As Filed Claims

We Claim

1. A recombinant penicillin V acylase enzyme represented by SEQ ID NO.1.
2. The enzyme as claimed in claim 1, wherein it is encoded by nucleotide sequence represented by SEQ ID NO.2, which is devoid of the part of the sequence encoding the periplasmic signal sequences.
3. The enzyme as claimed in claim 1, wherein the SEQ ID NO.2 is cloned in a vector and expressed in a host cell.
4. The enzyme as claimed in claim 1, wherein the vector is selected from the group consisting of pET28b and pET22b.
5. The enzyme as claimed in claim1, wherein the host cell is *E. coli* BL21.
6. The enzyme as claimed in claim 1, wherein it is stable at pH ranging from 3 to 6 and at temperature ranging from 20 to 50 degree C.
7. The enzyme as claimed in claim 1, wherein it is immobilized on solid substrate selected from the group consisting of alginate, acrylamide, and microporous beads.
8. A process for the preparation of the recombinant penicillin V acylase represented by SEQ ID NO.1 and claimed in claim 1, wherein the steps comprising:
   (a) Cloning the nucleotide sequence represented by SEQ ID NO. 2 in to a vector
   (b) Introducing the cloning vector of step (a) into a suitable host cell to express penicillin acylase in high yield, and
   (c) Purifying the expressed PVA enzyme by affinity column chromatography, wherein the prepared recombinant penicillin V acylase enzyme is secreted in the cytoplasmic fraction of the host cell.
9. The process as claimed in claim 8, wherein the yield of penicillin V acylase is in the range of 250 to 300 g/l of culture.
10. A recombinant bacterial strain of MCC0018.

Marked-up Copy of Claims

We Claim

1. A recombinant penicillin V acylase enzyme represented by SEQ ID NO.1 wherein recombinant penicillin V acylase ~~.~~

~~2. The enzyme as claimed in claim 1, wherein it~~ is encoded by nucleotide sequence represented by SEQ ID NO.2, which is devoid of the part of the sequence encoding the periplasmic signal sequences.

~~3.~~2. The enzyme as claimed in claim 1, wherein the SEQ ID NO.2 is cloned in a vector and expressed in a host cell.

~~4.~~3. The enzyme as claimed in claim 2~~1~~, wherein the vector is selected from the group consisting of pET28b and pET22b.

~~5.~~4. The enzyme as claimed in claim 2~~1~~, wherein the host cell is *E. coli* BL21.

~~6.~~5. The enzyme as claimed in claim 1, wherein ~~it~~ is the enzyme is stable at pH ranging from 3 to 6 and at temperature ranging from 20 to 50 degree C.

~~7.~~6. The enzyme as claimed in claim 1, wherein ~~it~~ is the enzyme immobilized on solid substrate selected from the group consisting of alginate, acrylamide, and microporous beads.

~~8.~~7. A process for the preparation of the recombinant penicillin V acylase represented by SEQ ID NO.1 and claimed in claim 1, wherein the steps comprising:

(a) Cloning the nucleotide sequence represented by SEQ ID NO. 2 in to a vector

(b) Introducing the cloning vector of step (a) into a suitable host cell to express penicillin acylase in high yield, and

(c) Purifying the expressed PVA enzyme by affinity column chromatography, wherein the prepared recombinant penicillin V acylase enzyme is secreted in the cytoplasmic fraction of the host cell.

~~9. The process as claimed in claim 8, wherein the yield of penicillin V acylase is in the range of 250 to 300 g/l of culture.~~

~~10.~~8. A recombinant bacterial strain having deposition no ~~.of~~ MCC0018 expressing recombinant penicillin Vacylase enzyme represented by SEQ ID NO.1, wherein

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recombinant penicillin V acylase is encoded by the nucleotide sequence represented by SEQ ID NO.2 , which is devoid of the sequence encoding the periplasmic signal sequence.

Clear Copy of Claims

We Claim

1. A recombinant penicillin V acylase enzyme represented by SEQ ID NO.1 wherein recombinant penicillin V acylase is encoded by nucleotide sequence represented by SEQ ID NO.2, which is devoid of the part of the sequence encoding the periplasmic signal sequences.
2. The enzyme as claimed in claim 1, wherein the SEQ ID NO.2 is cloned in a vector and expressed in a host cell.
3. The enzyme as claimed in claim 2, wherein the vector is selected from the group consisting of pET28b and pET22b.
4. The enzyme as claimed in claim 2, wherein the host cell is *E. coli* BL21.
5. The enzyme as claimed in claim 1, wherein is the enzyme is stable at pH ranging from 3 to 6 and at temperature ranging from 20 to 50 degree C.
6. The enzyme as claimed in claim 1, wherein is the enzyme immobilized on solid substrate selected from the group consisting of alginate, acrylamide, and microporous beads.
7. A process for the preparation of the recombinant penicillin V acylase represented by SEQ ID NO.1 and claimed in claim 1, wherein the steps comprising:
   (d) Cloning the nucleotide sequence represented by SEQ ID NO. 2 into a vector
   (e) Introducing the cloning vector of step (a) into a suitable host cell to express penicillin acylase in high yield, and
   (f) Purifying the expressed PVA enzyme by affinity column chromatography, wherein the prepared recombinant penicillin V acylase enzyme is secreted in the cytoplasmic fraction of the host cell.
8. A recombinant bacterial strain having deposition no. MCC0018 expressing recombinant penicillin V acylase enzyme represented by SEQ ID NO.1, wherein recombinant penicillin V acylase is encoded by the nucleotide sequence represented by SEQ ID NO.2, which is devoid of the sequence encoding the periplasmic signal sequence.