STEPS IN UNCOVERING THE KEY ENZYME IN THE DEGRADATION OF THE PYRIDINE RING IN THE *ARTHROBACTER NICOTINOVORANS*

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INTRODUCTION

Keywords: nicotine, pyridine ring, *Arthrobacter nicotinovorans*

The study of the degradation of the nicotine by the soil bacterium *Arthrobacter nicotinovorans* is still a work in progress but so far many discoveries have helped elucidating some of the steps that take place in its pathway.

Arthrobacter nicotinovorans is a Gram positive soil bacterium which is able to use nicotine as a source of C, N and energy. It produces a blue pigment when grown on nicotine as a result of the dimerisation of the hydroxylated pyridine ring. The mechanism of the degradation pyridine ring which is a part of the structure of the nicotine is still unknown. The elucidation of the entire process could prove to be useful in biotechnological applications in reducing the harmful effect that the nicotine found in the cigarettes' smoke or the liquid nicotine has on people and the environment.

The degradation steps from the 2, 6 dehydroxypseudooxynicotine to the end product nicotine blue (NB) , succinate and the proposed cleaving of the hydroxylated pyridine ring with the formation of α-ketoglutaric acid is shown in Fig 1.

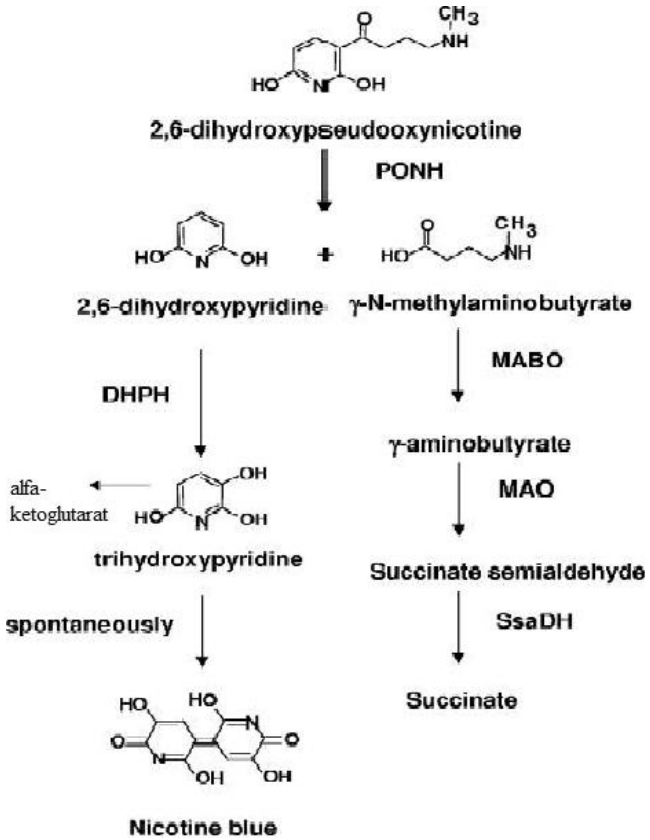


Fig. 1. Steps in nicotine catabolism leading from 2,6-dihydroxypseudooxynicotine to nicotine blue and succinate. The enzymes are PONH, DHPH, MABO, monoamine oxidaze (MAO), and succinate semialdehyde dehydrogenase (SsaDH). The proposed formation of α-ketoglutaric acid from trihydroxypiridine is indicated.

The hypothetical enzymes involved in the transformation from 2, 6 dyhydroxypyridine in α-ketoglutarat are all situated in the nic-gene cluster of the plasmid pAO1 and they are cleaving enzyme A (CEA), cleaving enzyme B (PKC), dyhydroxypyridinehydroxylase (DHPH) and Nitrilase.

Fig. 2. The nic-gene cluster from pAO1 from ***Arthrobacter nicotinovorans* , ORF 106 encodes CEA, ORF 310 encodes PKC and ORF 294 encodes nitrilase**

MATERIALS AND METHODS

The chemicals used were from the companies Roth, Sigma, Merck.

Bacterial strains and growth conditions. *Escherichia coli* XL-1 Blue was used as a host for plasmids and also as an expression strain. It was grown in Luria-Bertani (LB) medium supplemented with Ampiciline 100mg/ml at 37°C. *A. nicotinovorans* was grown at 30°C in citrate medium in the presence of Kanamycin 140 mg/ml.

PCR was performed to clone DHPH and CEA accordingly to the size of the genes. We have used 5 µl Buffer 10X Pfu Ultra Buffer, 1 µl dNTPs, 1 µl the corresponding forward primer and 1 µl of the corresponding reverse primer, 0.5 µl template *A. nicotinovorans* $pO1^+$ while type and 41.5 µl water. The program was designed according to the size of the gene and then the samples were run on a 1% agarose gel which contained ethidiumbromite in 1XTBE Buffer.

Determination of the activity of the degradation of the pyridine ring in extracts obtained from Arthobacter was determined by measuring the absorbance between 200 and 600 nm with Ultrospec 3100 Pro UV/visible spectrophotometer (Amersham)

HPLC of the deproteinated essays was performed with an instrument from Bischoff with a Hamilton PRP-X300 Ion Exclusion Column with acetonitrile as mobile phase and at 1.5mL/min flow.

For the expression and the purification of the proteins was used a Strep-tag column for the pASK-IBA vector from *E.coli* which were induced with 10 µl/100 ml culture of anhydrotetracycline 2mg/ml in dimethylformamid. The column was equilibrated with 2 CVs Buffer W , the supernatant from the culture was added, the column was washed 5 times with 1 CV Buffer W and then 6 times 0.5 CVs Buffer E were added for the elution of the binded protein. The fractions collected were run on a SDS-PAGE gel.

SDS-PAGE gels were NuPAGE Novex Bis-Tris Mini Gels from Invitrogen and were used for electrophoresis in the Xcell SureLock Mini-Cell. The sample is mixed with the Potassium-Phosphate Buffer in a total volume of 15 µl and then is added 5 µl NuPAGE LDS Sample Buffer (4X) and the samples are heated 10 minutes at 70^o C. The Upper (inner) chamber is filled with 200 ml 1X NuPAGE SDS Running Buffer. The running conditions are voltage: 200 V constant, time: 40 minutes and the expected current 100-125 mA/ gel (start) and 60-80 mA/gel (end).

The enzymes necessary for the cutting of the plasmid and the testing of the right clones were provided by New England Biolabs and were used according to the required specifications

RESULTS AND DISCUSSIONS

Extracts of *Arthrobacter nicotinovorans* were obtained by using the French Press cell from cultures grown in citrate medium over night (0.05% nicotine, 5% mineral salts and 0.2% Kanamycin 140mg/ml). In these extracts the disappearance of the 2, 6 dihydroxypyridine can be recorded by measuring the decrease in absorption at 315 nm. The formation of nicotine blue from dimerization of 2,6 dihydroxypyridine is prevented.

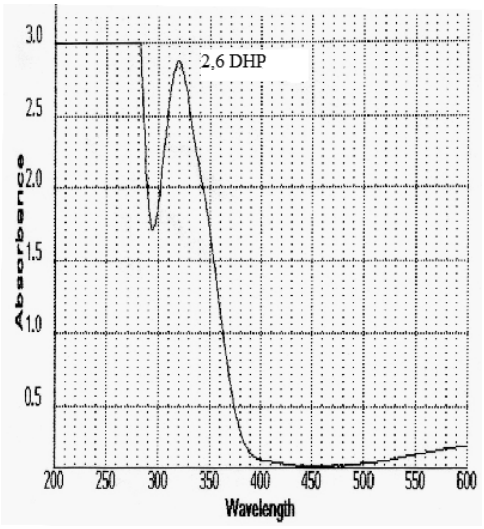


Fig. 3. *A. nicotinovorans* extract with 2,6 DHP added at the start of the experiment

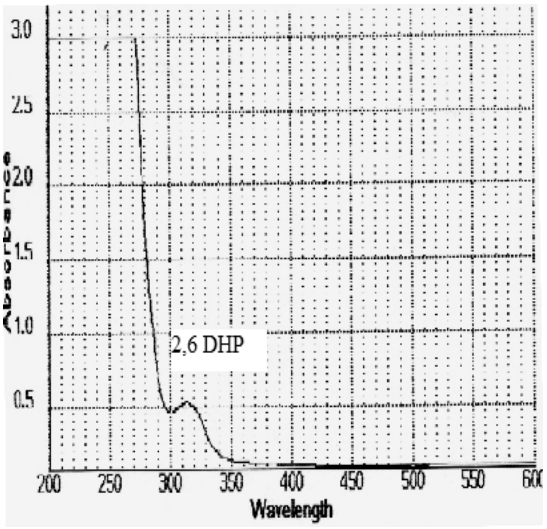


Fig. 4. *A.nicotinovorans* extract after 30 min. A strong decrease of the absorption of 2,6 DHP at 315nm can be observed

Extracts which were grown for more than 10 hours did not show the same decrease and also there could be seen the presence of the blue pigment which indicates the transformation of the 2, 6 dyhydroxypyridine into nicotine blue and not the cleaving of the ring as a result of the enzymes’ activity.

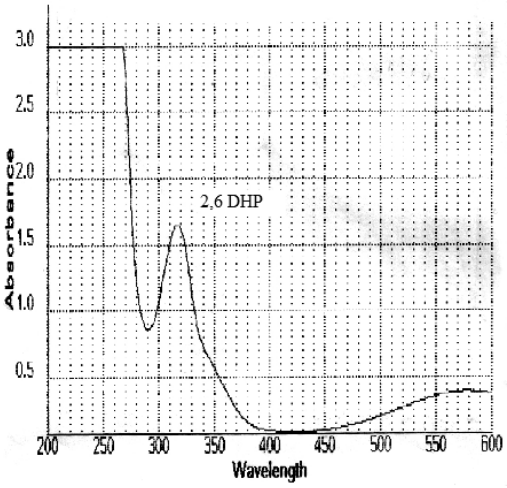


Fig. 5. A. nicotinovorans extract without cleaving activity. The formation of nicotine blue with an absorption maximum at 578 nm from 2,6 DHP can be observed

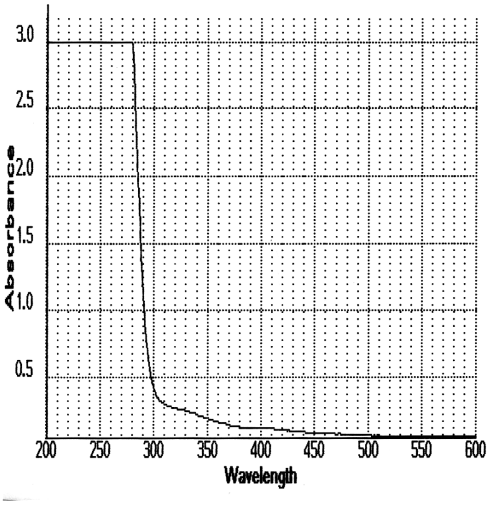


Fig. 6. Control with A.nicotinovorans extract without 2,6 DHP

The tests were repeated several times with the same result and therefore we can conclude that the cleaving takes place only in certain conditions regarding the time period of growth, the

concentration of the cells from the culture and the amount of Potassium-Phosphate Buffer pH=7,04 for resuspending the pallet used for the French Press. The smaller the volume of buffer, the bigger the concentration in cells and after the opening of the cells the bigger the concentration in enzymes as we can see in the picture.

Along with the reduction to disappearance of the peak for the 2,6 DHP without formation of BP, we can clearly see in tests run on the HPLC (50 µl injection) with the column for the organic acids the formation of α-ketoglutaric acid. This is a product that can be observed only in the extracts that have a high concentration of enzyme and that show the cleaving of the pyridine ring on the spectrophotometer.

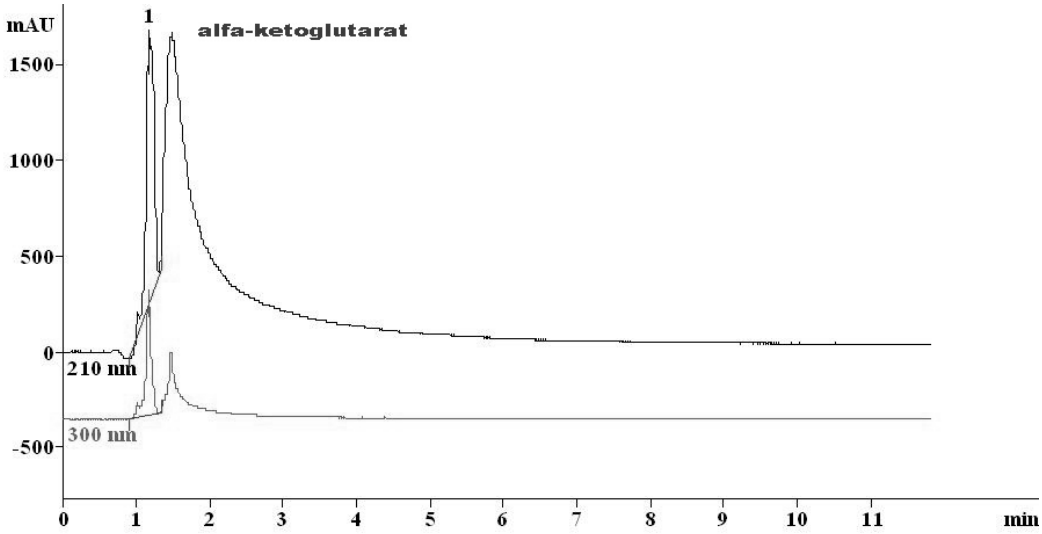


Fig.7. Sample on HPLC with the presence of α-ketoglutaric acid

Having the proofs that there is indeed a cleaving of the pyridine ring in the crude extracts, the next steps are directed to determine the enzyme which is responsible for this cleaving. For this we have cloned the nic-gene cluster and introduced it in the bacterium *Escherichia coli* and we have tested to see if in these new conditions *E.coli* can also use nicotine as a source of C and N. So far the results were inconclusive and more research is necessary in order to determine if this is a problem of conditions that *E.coli* need or this whole process is not compatible with the requirements of this bacterium.

Another direction of determination the role that each enzyme from the cluster plays in the degradation of the nicotine was to clone each gene from the nic-gene cluster in the IBA vector and to tests its functions by adding this enzyme in excess in in-vitro reaction we observing if there appears a decrease of the time needed to consume the 2, 6 DHP as a simple and easy way to measure this with the spectrophotometer.

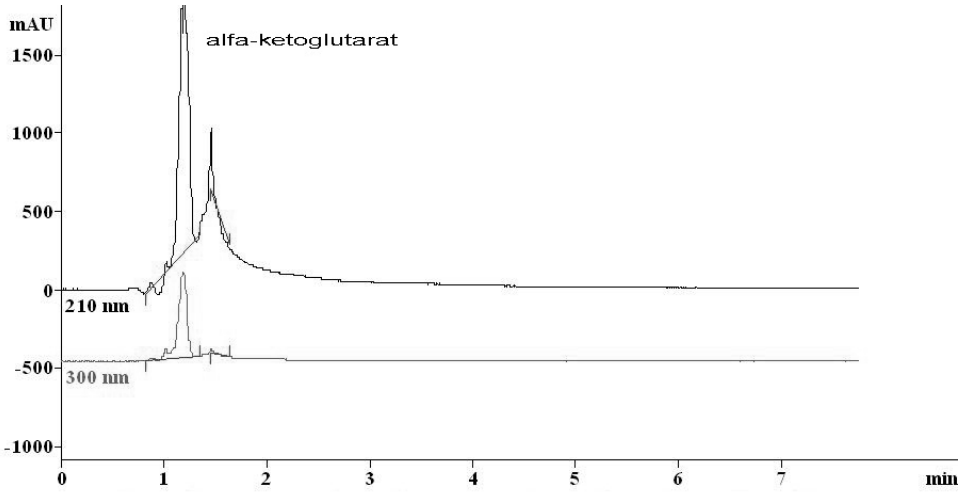


Fig. 8. Control run on HPLC that shows the presence of α-ketoglutaric acid but in a smaller quantity compared to the sample

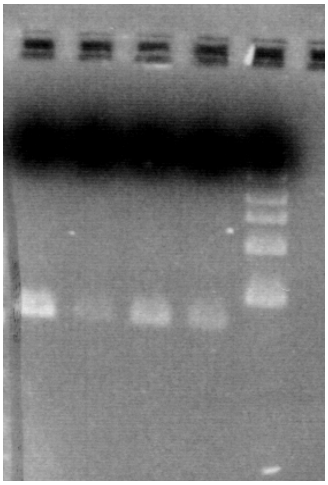


Fig.9.PCR for CEA lanes 1 to 4, lane 5 molecular weight 1kb marker

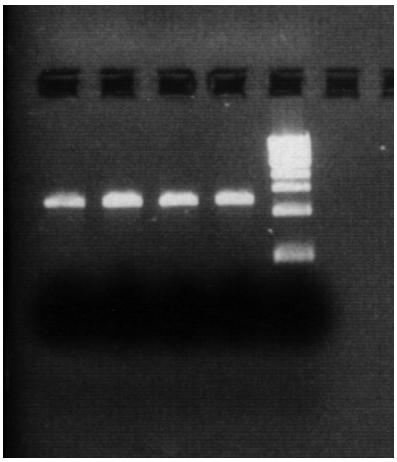


Fig. 10 PCR for DHPH lanes 1 to 4, lane 5 molecular weight 1kb marker

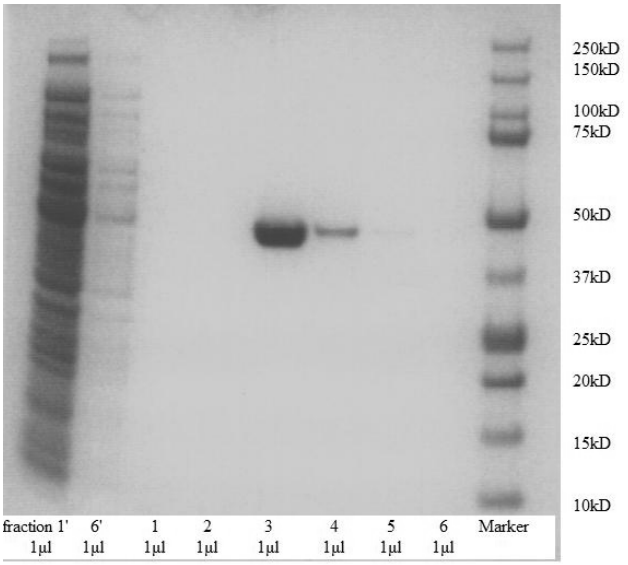


Fig. 11. The 6 fractions of DHPH collected from the Strep-tag column on SDS-PAGE, lanes 1 and 2 represent the first and the last fraction collected from the washing buffer of the column, lanes 1 to 8 represent fractions 1 to 6 collected with the elution buffer from the Strep-tag column. Lane 9 is precision plus protein standards marker.

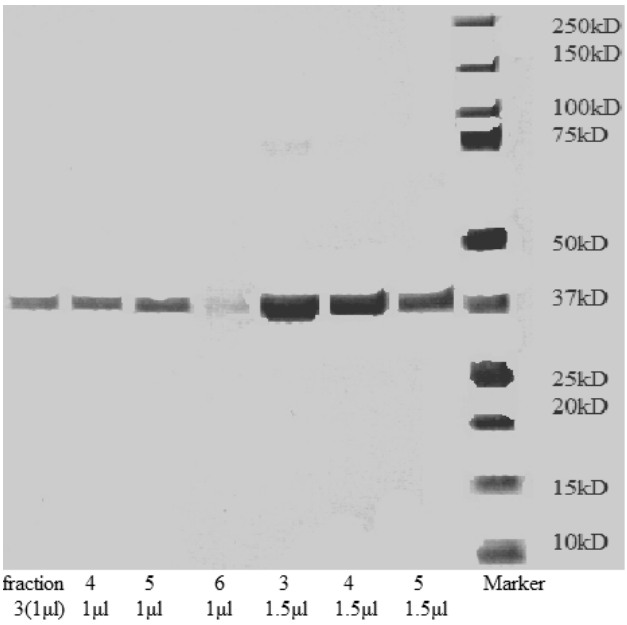


Fig. 12. The 6 fractions of Nitrilase collected from the Strep-tag column and run on SDS-PAGE, lane 1 to 4 contain 1 µl from of the fractions number 3, number 4, number 5 and number 6, lanes 5 to 8 contain 1.5 µl of the same fractions. Lane 9 is precise plus protein standards marker

Nitrilase is one of the enzymes which are believed to play an important role in the pathway of the degradation of the pyridine ring. This enzyme obtained from the nic-gene cluster is assumed to desaminate L-glutamic acid amide, and to form glutamic acid which acts as a substrate for the Glutamatdehydrogenase (GDH) that requires $NADP^+$. This reaction is relative easy to determine by obtaining Nitrilase by purification with a Strep-tag column. The extract comes from an overnight culture of *E.coli* with Nitrilase, grown in LB medium with 100mg/ml Ampiciline and which was opened by French Press. The enzyme obtained has a concentration of 3,6 µg/ml and is added (50 µl) with the substrate L-glutamic acid amide 10 mM (Sigma) in the presence of K-P Buffer pH=7,04 . The reaction is the continued by adding $NADP^+$ 10mM and GDH 0.1U/ml and measured at 340 nm in the spectrophotometer for 10 minutes.

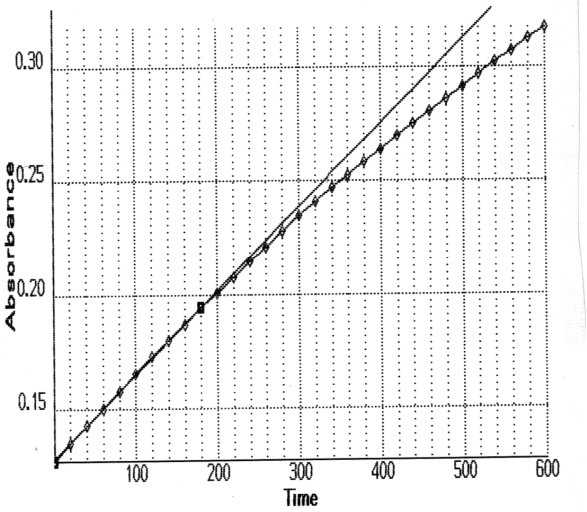


Fig. 13. Standard reaction of GDH with standard glutamic acid and $NADP^+$

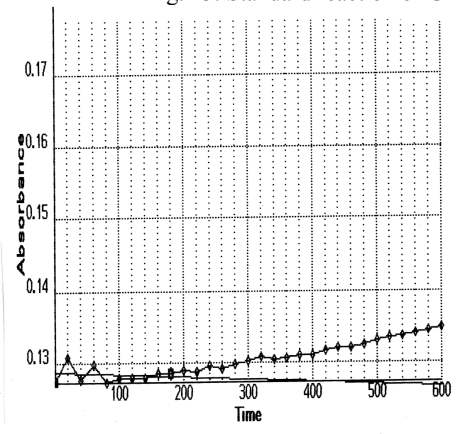


Fig. 14. Measuring the increase in NADPH in the reaction with GDH after 10 min

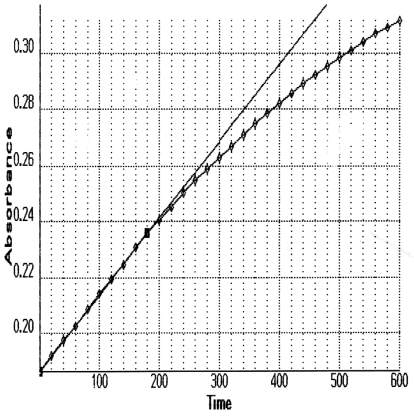


Fig. 15. Measurement of the increase of the NADPH in the reaction with GDH in the sample kept overnight with Nitrilase and the substrate

The activity of GDH is much more reduced, reflecting the low formation of glutamate from L-glutamic acid amide by nitrilase. Taking this into consideration we assume that α-ketoglutarat 5-amide is the right substrate of the proposed nitrilase, which could explain the low turnover of L-glutamic acid 1-amide into L-glutamic acid, but this remains to be tested on the specific substrate that this enzyme requires.

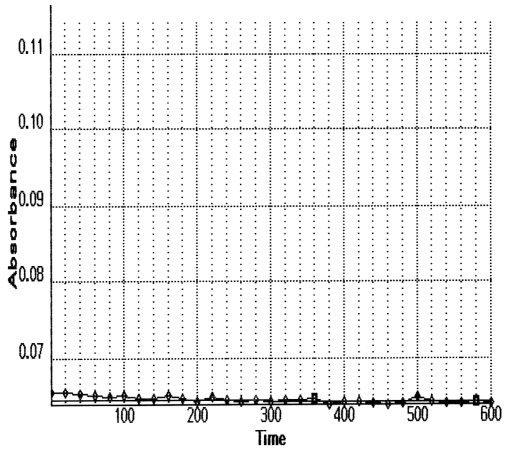


Fig.16 Control without Nitrilase

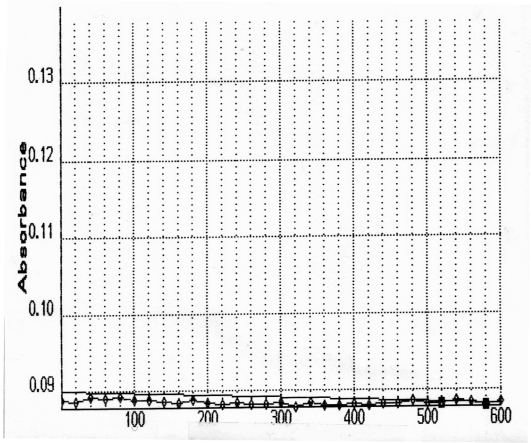
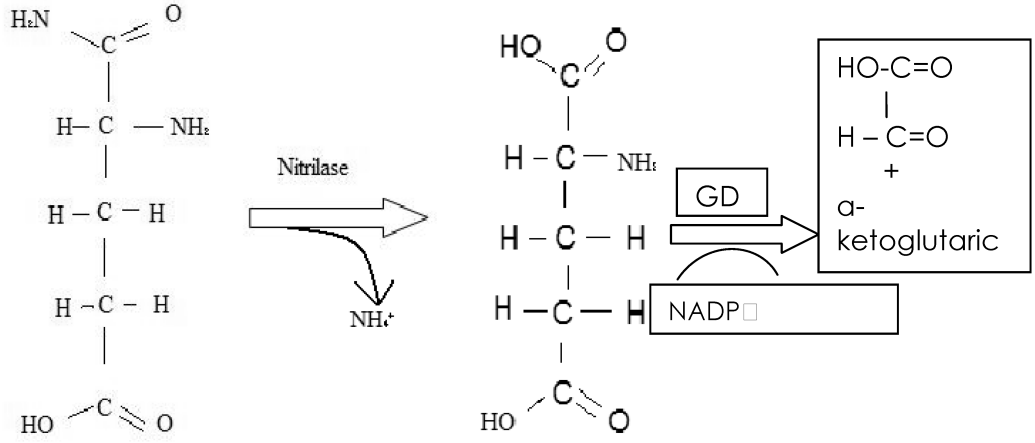


Fig. 17 Control without Nitrilase after 24h

The reaction is considered to be as in Fig.19 in the presence of the Nitrilase from the extract.



L- glutamic acid 1-amide L- glutamic acid (Glu)

Fig. 18 The assumed transformation of L-glutamic acid amide into the amino acid L-Glu in the presence of Nitrilase.

Tests using fresh extracts of *E. coli* containing one of the enzymes from the gene cluster (CEA, PKC, DHPH or Nitrilase) and adding the other enzymes purified by a Strep-tag column

were proved inconclusively. More research is needed to find out if here also is a problem of concentration or activity of the enzymes.

CONCLUSION

The process of identifying the enzyme responsible for the cleaving of the pyridine ring turns out to be more complicated than anticipated but with more work the solution can be found.

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