 Egypt. J. Chem. **Vol. 64,** No. 8 pp. 4369 - 4379 (2021)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Extracellular Hydrolysis of NAD$^+$ and its Secondary Metabolites by a new *Aspergillus sp*

Thanaa H. Ali*, **Latifa A. Mohamed**,

National Research Centre, Genetic Engineering and Biotechnology Research Division, Microbial Chemistry Dept., 33 El Bohouth St., Dokki, Cairo, P.O.12622, Egypt,

Abstract

In some organisms, the metabolism of extracellular nicotinamide adenine dinucleotide (NAD$^+$) plays an important role in purine salvage and uptake. Since filamentous fungi are used to degrade NAD and produce beneficial secondary metabolites, various enzymes, fermented foods and organic acids. Many strains belonging to filamentous fungi were screened for the enzymes production that catalyzed hydrolytic dephosphorylation and deamination of NAD and its secondary metabolites. A fungal isolate which was found to be the most prominent strain was identified genetically as *Aspergillus fumigatous*. Extracts of this new strain catalyzes the hydrolysis the ester bond between the two nucleotides of NAD by pyrophosphatase activity, which catalyzes NAD to nicotinamide mononucleotide (NMN) and adenosine monophosphate (AMP), at that point NMN and AMP are hydrolyzed tonicotinamide riboside (NR) and adenosine separately by 5'–nucleotidase activity This research reports also existence of deaminase activity hydrolyzed adenosine to inosine. The study demonstrated major enzymatic pathways involved in NAD degradationand identification of intermediates formed by *Aspergillus fumigatous*. Separation and partial purification of these enzymes and some kinetic properties of them were achieved.

Keywords: NAD$^+$ degradation, *Aspergillussp,* pyrophosphatases, deaminase

1. Introduction

Purine and pyrimidine nucleotides or nucleoside are those intracellular biomolecules the best known role of which is to serve as the monomeric precursors or units of deoxyribonucleic acids and ribonucleic acid [1-2]. Adenine nucleotides are also components of certain coenzymes, that has crucial roles in cellular energetic as the majority of the dehydrogenases which catalyze oxidation –reduction reactions and which involve electron transfer use NAD for this purpose, where as its degradation is a key element of important signaling pathways [3,4].

. A diphosphatase (pyrophosphatase), is performing an enzymatic activity towards NAD as favored substrate and giving NMN and AMP as products [5].Pyrophosphatase (EC 3.6.1.1) from a wide assortment of sources have been distinguished and classified**.** Soluble pyrophosphatases has been characterized in yeast [6], is able to catalyze NAD hydrolysis at alkaline reaction, and strongly inhibited by Fluoride. Among the prokaryotic NAD pyrophosphatases, (NadN) from *Haemophilus influenzae* and had two exercises: it hydrolyzes NAD to NMN and AMP by NAD pyrophosphatase action, at that point NMN and AMP are hydrolyzed to NR and Ado individually by 5'–nucleotidase activity[7]. In E. coli, cytoplasmic NudCand E. coli, UshA has been known as NAD(H) pyrophosphatase, which has the two enzymes action hydrolyzes NAD to nicotinamide mononucleotide (NMN) and adenosine monophosphate (AMP [8- 9]

Another way to cleave the purine and pyridine nucleotide particles is at the level of phosphate bond by means of hydrolytic phosphohydrolases. They are a family of multifunctional enzymes broadly disseminated in both plant and animal cells. Acid phosphatase produced by *Serratia* sp. has several applications, including plant growth promotion [10-11], waste remediation and metal recovery [12], antagonistic activity against plant pathogens [13] and hydroxylapatite biosnthysis environmental bioremediation and biosynthetic processes[14]. Acid phosphatases of *Saccharomyces cerevisiae* and *Aspergillus nidulans* have been extensively studied [15-16]. Acid phosphatase is detailed to be found within the cell wall and septa of hyphae

*Corresponding author e-mail: thanaa15@yahoo.com.; (Thanaa H. Ali).
Receive Date: 17 March 2021, Revise Date: 11 April 2021, Accept Date: 18 April 2021
DOI: 10.21608/EJCHEM.2021.68334.3491
©2021 National Information and Documentation Center (NIDOC)

as decided by cytochemical studies by transmission electron microscopy (TEM) [17].In some filamentous fungi, acid phosphatases involved in NAD hydrolysis to nicotinamide riboside and adenosine as have been characterized in our laboratory from *A. niger*[18], *A.orysae* [19-20]and *P. brevicompactum*[21]. The acid phosphatase, at the these previous fungal strains split ADP to AMP furthermore free inorganic phosphates. AMP, then hydrolyzed by the same enzyme, produces adenosine additionally Pi. Nonspecific debasement of NAD to nicotinamide riboside was recognized in Escherichia coli [22].

In this work, ribonucleosides such as adenosine, inosine and NR are final products. Resembling adenosine, inosine acting through adenosine receptors (ARs) applies a wide extend of anti-inflammatory and immunomodulatory impacts in vivo [23-25]. While NR, has shown promise for improving cardiovascular healthand as such administration of nicotinamide riboside supplemented-food product could also protect against axonal degeneration[26].Previous researches in filamentous fungi such *A. oryzae*, *P. brevicompactum* and *P. politans* that catalyzed deamination of adenosine to inosine and it was isolated and somewhat purified by Ali et al., [20], Mohamed et al. [27].

With the above notions in consideration, this study focuses on the metabolism of NAD and its derivatives through phosphate or ammonia hydrolyzing enzymes which has been identified and characterized from fungal sps. Further attempt has been made to purify and characterize the phosphohydrolase and the deaminase enzymes produced by the fungal isolates which may have potential biotechnological application.

2. Experimental

2.1. Materials

NAD, AMP, UMP, CMP, Adenosine, adenine, inosine, cytosine, guanine, cytidine, were purchased from Sigma Chemical Company, nicotinamide and acetamidewerepurchased from Merck. DEAE-cellulose was from Pharmacia Fine Chemicals. The proteinmolecular weight (MW) markers were purchased from Serva Electrophroesis GmbH D-69115 Heidelberg Carl-Benz-Str 7. All other reagents were prepared in MicrobialChemistry Department, National Research Centre.

2.2. Isolation and identification of the fungal organism

Among ten fungal isolates, the best isolate activity was selected was revived and grown on potato dextrose slant at 28 °C for 7days and stored at 4 °C. The morphological (color, texture appearance, and diameter of the colonies) and microscopic characteristics of the fungal isolate was performed by the Territorial Center for Mycology and Biotechnology, El-Azhar University. Fungal isolate DNA was extracted by the protocol of [28]. The primers used for the amplification and sequencing of 18S-rRNA encoding gene were widespread preliminaries (ITS1 and ITS4). The groupings of the ITS1 and ITS4 groundworks were 5'-TCCGTAGGTGAACCTGCGG-3' and 5'-TCCTCCGCTTATTGATATGC-3', respectively [29]. The PCR products were sequenced using specific purification kit (Accu Prep PCR DNA purification kit, k-30341, Bioneerorporation). The obtained sequence was aligned using the BLAST program (National Centre for Biotechnology Information) to find matches within the NCBI database [30]. 18S- rRNA quality part sequencing and recognizable proof of the confine 23were carried out utilizing Bhore strategy [31]

2.3. Preparation of identified fungal extracts

The 4 days old mats, developed on fluid altered potato dextrose medium containing per liter: 300 g of potato, 20 g dextrose at 28 °C, were collected by filtration, washed completely with distilled water and smudged dry with absorbent paper. The mats were at that point ground with cold washed sand in a chilled mortar and extricated with cold distilled water. The slurry in this way gotten was centrifuged at 1,5229 x g for 10 min and the supernatant was utilized as the rough protein planning

2.4. Thin layer chromatography (TLC) investigation

Enzymatic products of the reaction mixtures were tested by TLC on silica gel agreeing to a past strategy [32]. Reactions were done at appropriate condition contained 5 μmol NAD, 0.1 mg/mL somewhat purified protein. Reactions were e nded by including 5 μL of 2 M HCl, and centrifuged at 12,000 × g for 10 min. Spotted 1.0μL of tests onto TLC plates, created in a water/acetic corrosive /n–butanol (5: 3: 12; vol/vol/vol) blend, and visualized under UV at 254 nm

2.5.Phosphohydrolase assay

Phosphohydrolase (nonspecific orthophosphoric monoesterphosphohydrolase, EC 3.1.3.2) was determined by the method of Heninone and Lahti [33]using NAD as the substrate. One unit of

phosphatase activity is definedas the release of 1 µmol Pi at 1 min at 40C° at a pH of 3.5

2.6.Deaminase assay

NAD deaminase activity was decided as ammonia agreeing to the method depicted by Vogel 1961 [34]. Specific activity was expressed as lmol NH_3 liberated per mg protein per min, and a unit of enzyme action was characterized as the amount of enzyme required to produce 1 µM NH3 per min beneath the measure conditions. Protein concentration was decided concurring to Bradford (1976)[35] utilizing bovine serum albumin (BSA) as a standard. The protein content of the decontaminated enzyme divisions was determined by the UV absorbance agreeing to the strategy of Schleif and Wensink (1981)[36].

2.7. Separation and refinement of phosphohydrolase and aminohydrolase

Cold acetone (-15°C) was added to the unrefined extricate at acetone fractionation at concentration 0–33, 30–60, 60–75 and 75–83.5 % separately. Dialysis of this division was carried against cold distilled water for 3 h at around 7 °C. The dialyzed solution was loaded onto a DEAE-cellulose column(1.0x45 cm), pre-equilibrated with 0. 1 M Tris–acetate buffer at pH 6.0. Elution was carried at room temperature by batch-wise augmentations of 50 ml portion

2.8.Optimal pH and temperature stability

The ideal pH of the purified enzymes (phosphohydrolase or deaminase) was decided by performing the enzyme measure in several pH values extending (pH 1.0–9.0). The pH stability of the purified protein was inspected by measuring the remaining action after incubating the enzyme at each craved pH for 30 min. The ideal temperatures of the two over enzymes were determined within the temperature extend of 20–80°. The thermal stability was inspected by measuring the residual action after brooding the enzyme 60 °C.

2.9. Influnce of expansion of some compounds on the purified enzyme activities

The effects of a some added substances on phosphohydrolase and deaminase activities were examined. The purified proteins were pre-incubated with 10, 20 mM compounds at 40°C for 30 min, Tris–acetate buffer (80 mM, pH 3.5). The metal particles were: Mg2+, Ag2+, Mn2+,Hg2+,Cu2+and MoO42-. Some anion such as F- and N3- were determined. The enzymes activities was decided by the standard measure as portrayed over using NAD as substrate

2.10. Substrate specificity

Substrate specificity was explored by replacing NAD in the standard measure blend with an equal concentration of the following represent active phosphorylated or aminated compounds: NAD, AMP, UMP, CMP, adenosine, cytosine, guanosine and cytidine

2.11. Kinetic Data investigation.

Data fitting was performed agreeing to the Michaelis-Menten equation:

$Kcat=V max/Et$

Et= total enzyme concentration

to fit the experimental data and to obtain gauges of the active parameters utilize was made of the excel program. All tests were rehashed at least 3 times, results are displayed as mean ± standard deviation.

3. Results and discussion

3.1.Quantitative analysis

Ten different fungal strains were screened for their intracellular phosphohydrolase or deaminase or glycohydrolase activity involving in NAD degradation. All these fungal isolates gave activity in acid or in alkaline medium with variable degrees at the phosphate hydrolysis after 4 days of incubation. Results of the experiment (Table 1) showed that isolate No. E23 was the highest production exhibiting the highest phosphate and ammonia hydrolysis activity at acidic reaction. No hydrolytic cleavage of N-glycosidic linkage of NAD might be identified within the past reaction.

3.2. Microscopic and molecular identification of the fungal isolateE23

Fungal taxonomy is traditionally built on relative morphological features [36]. The most efficient pyrophosphatase, phosphatase and deaminase producer, isolate E23 was identified based on morphological features. The microscopic examination revealed that our strain was indeed fungus with sub spherical conidia that measured 3.0 µm with blue green conidial heads and the conidiophores were 12µm in diameter as cleared in Fig.1. The sequence data of nucleotides of 18SrRNA genes were deposited to GenBank with accession number KX507082.1

Fig. 1 Accordingly the specimen is: *Aspergillus fumigatus* var. ellipticus

Aspergillus fumigatus internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

```
Query  42   GCTT-CCCGCCATCAATTACGCTCGGACAGGCATGCCCCCCGCGAATACCAGGGGGCGCA
100
            |||| |||| || |||| |||||||||||||||||||||||| |||||||||||||||||
Sbjct  254  GCTTGCCCGGCAGCAATGACGCTCGGACAGGCATGCCCCCCG-GAATACCAGGGGGCGCA
196

Query  101  ATGTGCTTTCAAAGACTCAATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATT
160
            |||||| ||||||||||| |||||||||||||||||||||||||||||||||||||||||
Sbjct  195  ATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATT
136

Query  161  TCCCTCCGTTCTTCATCCATGCCGGAACCAAGACATCCGTTGTTGAAAGTTTTAACTGAT
220
            || || ||||||||||| ||||||||||||||| ||||||||||||||||||||||||||
Sbjct  135  TCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTGAT
76

Query  221  TACTATAATCAACTCAAACTACATACTTTCAAAA  254
            ||| |||||||||||| ||| |||||||||||||
Sbjct  75   TACGATAATCAACTCAGACTGCATACTTTCAAAA  42
```

Table 1 *Quantitative analysis of enzymes degrading NAD*

Organism Isolates	Specific activity of phosphatase at pH4	pH8	Specific activity of deaminase at pH4	pH8
Isolate	0.02	0.12	0	0
Isolate 8	0.2	0.14	0.05	0.1
Isolate 10	0.05	0	0	0.07
Isolate 23	**0.81**	**0.26**	**0.1**	**0.45**
Isolate 25	0.18	0.09	0.08	0.04
Isolate 30	0.08	0.01	0.01	0.074
Isolate 31	0.17	0.04	0.02	0.09
Isolate 33	0.092	0.0	0.0	0.02
Isolate 34	0.041	0.02	0.0	0.09
Isolate 39	0.097	0.05	0.01	0.1

3.3. Separation and Purification of NAD degrading Enzymes

The separation of NAD phosphohydrolase and NAD aminohydrolase was described under ''Materials and methods'' section. A outline of the distinctive protein separation steps carried out to purify the two proteins that degrade NAD as substrate to screen the enzyme action all through the purification procedures. Acetone fractionation anion exchange chromatography using DEAE-cellulose. Figure.2 shows that during all purification steps, at NAD degradation, NAD pyrophosphatase activity should be associated with 5'–nucleotidase activity andwere detected in protein fractions with one peak gave partially purified (132-fold) with a yield of 64.6 with molecular weight about 64 kDaand, the activities of NAD deaminating recorded in anther peak gave partially purified (58fold) enzyme with a yield of 55with molecular weight about 96 kDa.

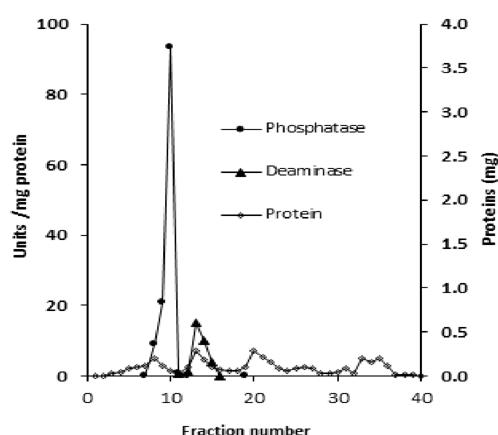

Fig. 2 Separation of NAD aminohydrolase from NAD phosphohydrolase by Sephadex G-100

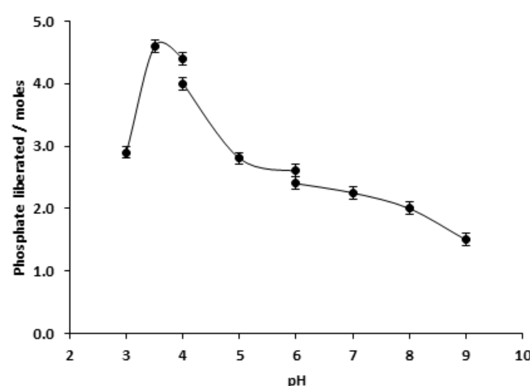

Fig. 3 pH profile of phosphohydrolase activity

3.4. Optimal pH and temperature stability of the enzymes

The impact of pH on the phosphohydrolase activity was shown ideal action at pH3.5 (Fig. 3) and appeared stability within a pH range of 3.0–5.0 (data not shown). At pH 8.0, the phosphohydrolase loses almost 80% of its activity. The ideal temperature for the phosphohydrolase was 60°C (Fig. 4). The enzyme was steady over a temperature extend of 30–70 °C (see Fig. 4). Where as the deaminase having ideal action at pH 6.0 and appeared stability within a pH extend of 4.0–7.0 (Fig. 5). At pH 8.0, the deaminase loses 45 % of its activity. The ideal temperature for the deaminase was 70 °C (data not appeared)

3.5. Identification of intermediates formed in NADdegrading reaction

NAD degradation reaction by TLC clearly demonstrated total dephosphorylation of NAD that NAD was totally expended, but there was clearly no accumulation of either AMP or NMN. Instep, the presence of nicotinamide riboside, adenosine and inosine was apparent for

those paths with total utilization of NAD (data not appeared). It was known that NAD can be hydrolyzed to form NMN and AMP by NAD pyrophosphatase as what has been detailed in E. coli[9]. In any case, TLC comes about proposed that A. fumigatous catalyzed further hydrolysis of NMN and AMP into NmR and Ado, respectively, by 5'–nucleotidase action, at that point adenosine can deaminated to adenine. This pathway may speak to the most limited and most energy-saving way that can be included in NAD degradation as appeared in (Fig. 6).

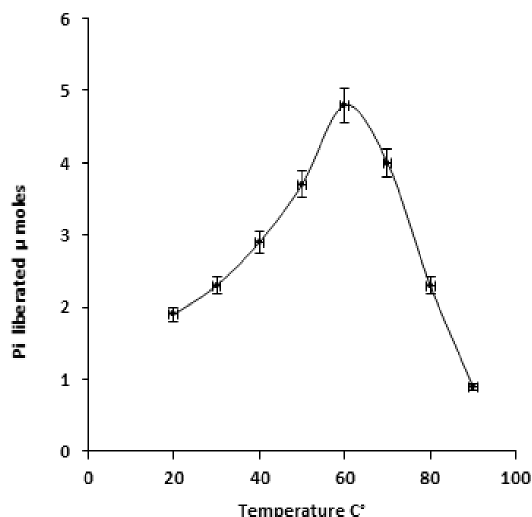

Fig. 4 Influence of temperature on the phosphohydrolase activity

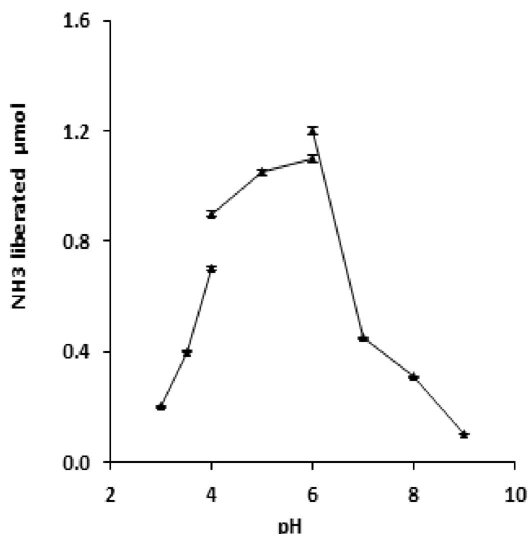

Fig. 5 pH dependance of the aminohydrolase activity

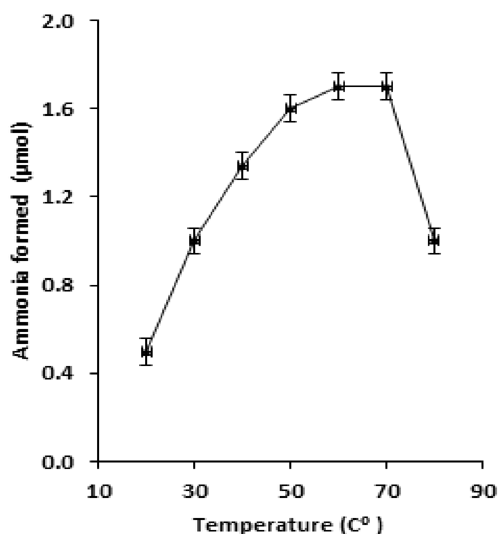

Fig. 6 Effect of temperature on *A. fumigatous* aminohydrolase

Additionally, it appeared that A. fumigatous had much higher 5'–nucleotidase action than pyrophosphatase and deaminase activities. This NAD dephosphorylating pathway is comparable to the closely resembling pathways influenced by each of the pyrophosphatase of *Saccharomyces cerevisiae*[39], *Escherichia coli* [39, 9]and *Haemophilus influenza*[40].

In accordance with the assumption presence of 5'–nucleotidase that cleavage MNM and AMP to nicotinamide riboside and adenosine as products chromatographically detected in each of the different reaction mixtures.This 5'–nucleotidase has acidic natured activity like *A oryzae* acid phosphatase [20] but it differs from *Penicillium brevicompactum*, *Escherichia coli* enzymes [21, 9] that the two later organisms having optimum activity at alkaline reactions. Appearance of inosine and adenosine in chromatographic examination illustrated the partial d eamination of adenosine as the results has been found in extracts of *A. niger*[18], *A. terreus*[41]and *A. oryzae* [19] *and Penicillium brevicompactum*, [21]. From chromatographic analysis, No ability of the pure aminohydrolase to deaminate the amide group of each nicotinamide or nicotinamide riboside

3.6.Effect of some compounds on phosphohydrolase activity

As it shows up from Table 2, Mg2+ and Ca2+ decently actuated the enzyme as about 30% increase in the enzyme action. $Fe^{3+}$and Cu^{2+} inhibited the enzyme activity about 50%. Fluoride, azide and molybdatehave considerable inhibitory effect as they caused

about 90% inhibition. The result confirms the metalloenzymatic nature of the phosphatase superfamily has solid reliance on divalentcations. Maximal action of the enzyme was watched with theMg2+ion at the concentration of 0.2 mM toward pyrophosphatase. The comes about support the conclusion that Mg,PPi is the substrate which there's a high-affinity Mg2+ binding site [42-43].The hydrolysis of dinucleotide pyrophosphates requires divalent metal ions and yields two mononucleoside 5'-phosphates, has been illustrated in *Escherichia coli* [9]

Table 2 Effect of some compounds on phosphohydrolase activity

Compounds	Activity (%)
Control	100
Mg^{2+}	135
Ca^{2+}	130
Zn^{2+}	100
Cu^{2+}	41.0
N_3	10.0
MoO_4	13.0
Fe^3	32.0
F	10.0

3.7. Substrate specificity, catalytic efficiency and kinetic perfection of the purified enzymes

Kinetic parameters of *A. fumigatous* phosphohydrolase catalysis were estimated using NAD, AMP, CMP, UMP and pheny phosphate, as substrates (Table 3). Whereas Km values were near for all of these substrates, kcat values for AMP and was orders of magnitude higher than those for NAD. The kcat/Km value for AMP was generally one order of magnitude higher than those for NAD, demonstrating that *A. fumigatous* phosphhydrolase had considerably higher catalytic productivity for phosphate hydrolysis than pyrophosphate hydrolysis.

The *Km* values of *A. fumigatous* phosphohydrolase for NAD (1.91 $x10^{-4}M$) was considerably lower, demonstrating enzyme has much more grounded binding affinity toward NAD compared to other pyrophosphatases. For NAD degradation, the foremost effective pyrophosphatase was was *A. fumigatous* with a *kcat/Km* value of 3.82 x 10 $^{-4}$ M^{-1} $^{s-}$which was considerably higher than both of *E. coli*, NudC and *E. coli* UshA of that they had low catalytic efficiencies with NAD equal (2.9×10–4μM^{-1} $^{s-1}$) and (1.4 μM^{-1} $^{s-1}$), [8-9] respectively.

Different specificity of the aminohydrolase action Data of Table 3 appears that the pure amidohydrolase displayed activities as it were with nucleotides containing amino groups and their corresponding nucleosides and bases out of the tested compounds containing amide groups such as nicotinamide and acetamide. These comes about shown that this enzyme to be classified as deaminase enzyme that evacuating the amino group from adenosine, whereas it has no action toward amide group of nicotinamide, and this result demonstrated the comes about gotten in Table 3, broad specificity of the *A. fumigatous* aminohydrolase has been recommended amid this work, as, in addition to NAD, the enzyme preparation had the capacity to hydrolyze ammonia from other purine and pyridine compounds. The appearance of ammonia in NAD reaction with crude enzymes forms due to deamination of adenosine which was formed due to dephosphorylation of NAD by action of pyrophosphatase and 5'–nucleotidase activities.

Results of Table 3 show that kcat*/Km* value of adenosine 1.23 x 10 $^{-5}$ M^{-1} $^{s-}$ which is less than *kcat/Km* value in case of NAD and AMP. In addition *kcat/Km* value of adenosine was also less than the other purine and pyrimidine nucleoside degradation in both of *A. oryzae and P.brevi compactum*[20-21].Therefore, it becomes more efficient, and hence more products are generated at a faster rate than in other microorganisms. The enzyme catalyzes the hydrolysis of a wide run of mononucleotide phosphates, but interestingly lean towards the adenosine..

Interestingly, the present study found that extracts of an *A. fumigatous* deaminase had high hydrolytic activities over a wide range of substrates, such as AMP, CMP and UMP.

This NAD degradation pathway is comparable to the practically equivalent to pathways influenced by each of the pyrophosphatase *Saccharomyces cerevisiae, Escherichia coli* and *Haemophilus influenza*[38-40] in having pyrophosphatase and 5'–nucleotidase activities and the same pathway *while A. fumigatous* extracts contain also pyrophosphatase ,5'–nucleotidase and deaminase which convert adenosine to inosine as extracts which acts on this particle. Inclusion of these two already detailed pathways in NAD recycling has been illustrated in *Escherichia coli* and *Salmonella typhimurium*[9], [7]respectively

Our comes about and past information [39-9] demonstrated that A. fumigatous and E. coli UshA was indiscriminate (pyro)phosphatase protein which its NAD pyrophosphatase activity was recognizably lower than 5'–nucleotidase action. This activity profile may be beneficial for cells to encourage utilization of extracellular supplements beneath different situation.

For biotechnological application such as whole–cell biocatalysis, in any case, the expulsion of UshA could progress extracellular NAD solidness and cell growth

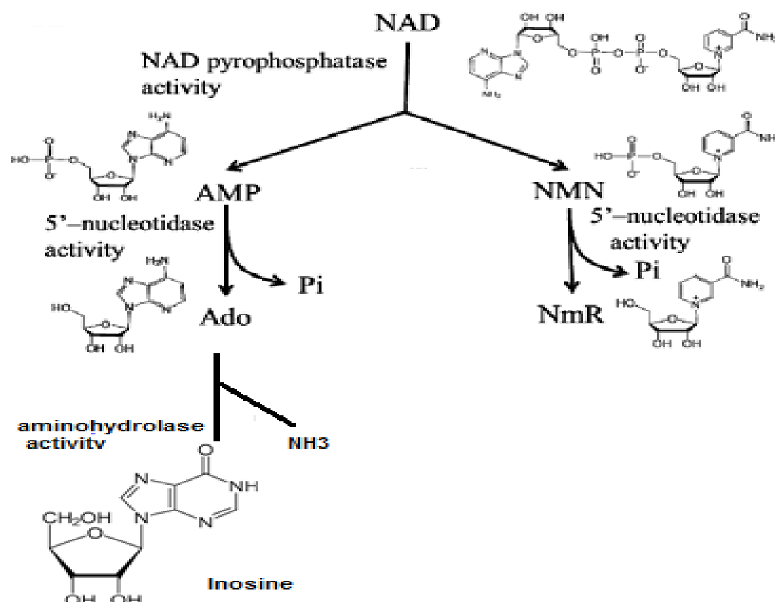

Fig.7 NAD degrading enzymes activity. Proposed NAD degradation paths by*A. Fumigatous* activity . Abbreviations: NAD, nicotinamide adenine dinucleotide; AMP, adenosine monophosphate; Ado, adenosine; NMN, nicotinamide mononucleotide; Ino, inosine

Table 3. Substrate specificity, catalytic efficiency and kinetic perfection of the partially purified acid phosphatase and deaminase activities

Enzyme	Km (M^{-1})	Vmax	kcat(s^{-1})	kcat/Km $M^{-1}s^{-1}$
Acid phosphatase				
NAD	19.14 10^{-3}	13.98601399	730.5669	3.82E+04
AMP	12.56 10^{-3}	6.756756757	537.7211	4.28E+04
CMP	7.61 10^{-3}	3.65497076	480.2382	6.31E+04
phph	6.59 10^{-3}	3.75516335	569.3464	8.63E+04
UMP	0	0	0	0
Aminohydrolase				
NAD	7.901506913	1.55351872	196.6104361	2.49E+04
AMP	11.98960573	3.584229391	298.944725	2.49E+04
Adenosine	3.816403446	1.794687724	470.2562897	1.23E+05
Cytidine	1.636377025	0.490918017	300.003	1.83E+05
Guanose	8.853333333	1.731601732	195.587545	2.21E+04
Nicotinamide	0	0	0	0
Acetamide	0	0	0	0
L-glutamine	0	0	0	0

4. Conclusion

The remarkable findings during the present study are the non previously recognized extents and modes of NAD degradations by extracts of an organism. These findings were rather unexpected especially with A. *Fumigatous* extracts, as, previously reported studies showed that extracts of an A. *Fumigatous* strain from the observed properties of the two pyrophosphatase of the experimental *E.coli* strain, it appears that they resemble each other in the way by which they dephosphorylate NAD to

adenosine and NR, in being orthophosphate- non repressible enzymes and in being not true phosphomonoesterases.

This last property was based on their abilities to catalyze hydrolytic cleavage of the pyrophosphate linkage of ADP and the internal ester linkage between NR and ADP, in addition to the true phosphate ester linkages of NADP+, AMP and NMN. However, the two enzymes were dissimilar in their molecular size (elution diagram), in their responses to the effects of different factors such as high temperature (in presence or absence of the substrate), dialysis of the extracts and addition of possible inhibitors and activators to the reaction mixtures Degradation of both NADP+ and NAD+ by extracts of the experimental A.niger strain over a wide range of pH values might make it rather hard to assay for dehydrogenases requiring any of these two coenzymes as an electron acceptor.

However, this degradation can be avoided through inactivation or inhibition of the phosphate releasing enzymes on condition that these treatments do not negatively affect the dehydrogenases under investigation.

However, during the present investigation NADP+ was degraded via the The present work demonstrated the occurrence of two alkaline phosphatases, aminohydrolase and glycohydrolase in P. brevicompactum NRC 829. These enzymes are involved in NAD degradation. Purification and separation showed high aminohydrolase activity with the catalytic efficiencies for hydrolysis of NAD and adenosine at 1.9 and 1.8 lM -1s -1, respectively. These results significantly enriched our understanding on NAD metabolism and should facilitate many applications including designing redox biocatalysts.

5. Conflicts of interest

The authors declare that they have no competing interest.

6. Acknowledgement

This study was supported by the National Research Centre, Cairo, Egypt

7. References

[1] C. Cantó, K.J. Menzies, J. Auwerx, NAD(+) Metabolism and the Control of Energy Homeostasis: A Balancing Act between Mitochondria and the Nucleus, Cell Metab., 22 (2015) 31-53.

[2] K. Yaku, K. Okabe, T. Nakagawa, NAD metabolism: Implications in aging and longevity, Ageing research reviews, 47 (2018) 1-17.

[3] R.H. Houtkooper, C. Cantó, R.J. Wanders, J. Auwerx, The secret life of NAD+: an old metabolite controlling new metabolic signaling pathways, Endocr. Rev., 31 (2010) 194-223.

[4] G. Magni, A. Amici, M. Emanuelli, G. Orsomando, N. Raffaelli, S. Ruggieri, Enzymology of NAD+ homeostasis in man, Cell. Mol. Life Sci., 61 (2004) 19-34.

[5] E. Fouquerel, R.W. Sobol, ARTD1 (PARP1) activation and NAD(+) in DNA repair and cell death, DNA Repair (Amst), 23 (2014) 27-32.

[6] T. Croft, P. Venkatakrishnan, S.J. Lin, NAD(+) Metabolism and Regulation: Lessons From Yeast, Biomolecules, 10 (2020).

[7] D. Ji, L. Wang, S. Hou, W. Liu, J. Wang, Q. Wang, Z.K. Zhao, Creation of bioorthogonal redox systems depending on nicotinamide flucytosine dinucleotide, J. Am. Chem. Soc., 133 (2011) 20857-20862.

[8] D.N. Frick, M.J. Bessman, Cloning, purification, and properties of a novel NADH pyrophosphatase. Evidence for a nucleotide pyrophosphatase catalytic domain in MutT-like enzymes, J. Biol. Chem., 270 (1995) 1529-1534.

[9] L. Wang, D. Yu, Strategy for Efficient Importing of NAD Analogs, bioRxiv, (2018) 480269.

[10] M. Schoebitz, C. Ceballos, L. Ciamp, Effect of immobilized phosphate solubilizing bacteria on wheat growth and phosphate uptake, Journal of soil science and plant nutrition, 13 (2013) 1-10.

[11] M.B. Farhat, S. Taktek, H. Chouayekh, Encapsulation in alginate enhanced the plant growth promoting activities of two phosphate solubilizing bacteria isolated from the phosphate mine of Gafsa, Net Journal of Agricultural Science, 2 (2014) 131-139.

[12] L.E. Macaskie, K.M. Bonthrone, P. Yong, D.T. Goddard, Enzymically mediated bioprecipitation of uranium by a Citrobacter sp.: a concerted role for exocellular lipopolysaccharide and associated phosphatase in biomineral formation, Microbiology, 146 (2000) 1855-1867.

[13] U. Chakraborty, B. Chakraborty, M. Basnet, Plant growth promotion and induction of resistance in Camellia sinensis by Bacillus megaterium, J. Basic Microbiol., 46 (2006) 186-195.

[14] R. Sammon, T. Hikov, E. Radeva, R. Presker, D. Mitev, L. Pramatarova, Biomineralisation on the composites of silicon-based polymer and nanodiamond particles by a species of Serratia bacteria, Bulgarian Journal of Physics, 41 (2014).

[15] M. Schweingruber, E. Edenharter, A. Zurlinden, K. Stockmaier, Regulation of pho1-encoded acid phosphatase of Schizosaccharomyces pombe by adenine and phosphate, Curr. Genet., 22 (1992) 289-292.

[16] H.N. Arst, C.R. Bailey, H.A. Penfold, A possible role for acid phosphatase in γ-amino-n-butyrate uptake in Aspergillus nidulans, Arch. Microbiol., 125 (1980) 153-158.
[17] F. Gutierrez-Miceli, M. Rodriguez-Mendiola, N. Ochoa-Alejo, R. Méndez-Salas, C. Arias-Castro, L. Dendooven, Sucrose accumulation and enzyme activities in callus culture of sugarcane, Biol. Plant., 49 (2005) 475-479.
[18] T. Elzainy, T. Ali, Pathways of NADP+ and NAD+ degradation by Aspergillus niger extracts, Ann. Microbiol., 50 (2000) 65-75.
[19] T. Ali, B. Haroun, A. Tantawy, Optimization of culture conditions for the production of NAD-degrading enzymes by Aspergillus oryzae NRRL 447, Adv. Food Sci., 34 (2012) 195-201.
[20] T.H. Ali, N.H. Ali, B.M. Haroun, A.E. Tantawy, Purification and partial characterization of NAD aminohydrolase from Aspergillus oryzae NRRL447, World Journal of Microbiology and Biotechnology, 30 (2014) 819-825.
[21] T.H. Ali, D.H. El-Ghonemy, Purification and characterization of the enzymes involved in nicotinamide adenine dinucleotide degradation by Penicillium brevicompactum NRC 829, 3 Biotech, 6 (2016) 34.
[22] G. Wang, T. Han, D. Nijhawan, P. Theodoropoulos, J. Naidoo, S. Yadavalli, H. Mirzaei, A.A. Pieper, J.M. Ready, S.L. McKnight, P7C3 neuroprotective chemicals function by activating the rate-limiting enzyme in NAD salvage, Cell, 158 (2014) 1324-1334.
[23] A.A. Welihinda, M. Kaur, K. Greene, Y. Zhai, E.P. Amento, The adenosine metabolite inosine is a functional agonist of the adenosine A2A receptor with a unique signaling bias, Cell. Signal., 28 (2016) 552-560.
[24] A.A. Welihinda, M. Kaur, K.S. Raveendran, E.P. Amento, Enhancement of inosine-mediated A2AR signaling through positive allosteric modulation, Cell. Signal., 42 (2018) 227-235.
[25] F. Hajizadeh, A. Masjedi, S.H. Asl, F.K. Kiani, M. Peydaveisi, G. Ghalamfarsa, F. Jadidi-Niaragh, A. Sevbitov, Adenosine and adenosine receptors in colorectal cancer, Int. Immunopharmacol., 87 (2020) 106853.
[26] W. Tempel, W.M. Rabeh, K.L. Bogan, P. Belenky, M. Wojcik, H.F. Seidle, L. Nedyalkova, T. Yang, A.A. Sauve, H.-W. Park, Nicotinamide riboside kinase structures reveal new pathways to NAD+, PLoS Biol., 5 (2007) e263.
[27] L.A. Mohamed, N.H. Ali, T.H. Ali, Biodegradation of mononucleotides to nucleosides and free bases, Afr. J. Microbiol. Res., 3 (2009) 613-621.
[28] K. Abd-Elsalam, A. Asran-Amal, A. El-Samawaty, Isolation of high-quality DNA from cotton and its fungal pathogens, J. Plant Dis. Prot., 114 (2007) 113-116.
[29] T.J. White, T. Bruns, S. Lee, J. Taylor, Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, PCR protocols: a guide to methods and applications, 18 (1990) 315-322.
[30] R. Yehia, E. Ali, A. Al-Zahrani, Feasibility of oleaginous fungi isolated from soil samples of Saudi Arabia for mycodiesel production, Appl. Biochem. Microbiol., 53 (2017) 94-100.
[31] S.J. Bhore, N. Ravichantar, C.Y. Loh, Screening of endophytic bacteria isolated from leaves of Sambung Nyawa [Gynura procumbens (Lour.) Merr.] for cytokinin-like compounds, Bioinformation, 5 (2010) 191.
[32] K.-S. Lee, S.B. Song, K.E. Kim, Y.-H. Kim, S.-K. Kim, B.H. Kho, D.-K. Ko, Y.-K. Choi, Y.-K. Lee, C.-K. Kim, Cloning and characterization of the UDP-sugar hydrolase gene (ushA) of Enterobacter aerogenes IFO 12010, Biochem. Biophys. Res. Commun., 269 (2000) 526-531.
[33] J.K. Heinonen, Biological role of inorganic pyrophosphate, Springer Science & Business Media, 2001.
[34] I. Lansdorp-Vogelaar, K.M. Kuntz, A.B. Knudsen, J.A. Wilschut, A.G. Zauber, M. van Ballegooijen, Stool DNA testing to screen for colorectal cancer in the Medicare population: a cost-effectiveness analysis, Ann. Intern. Med., 153 (2010) 368-377.
[35] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem., 72 (1976) 248-254.
[36] R.F. Schleif, P.C. Wensink, Working with proteins, in: Practical methods in molecular biology, Springer, 1981, pp. 61-88.
[37] J.-w. Zhang, R.-y. Zeng, Molecular cloning and expression of a cold-adapted lipase gene from an Antarctic deep sea psychrotrophic bacterium Pseudomonas sp. 7323, Mar. Biotechnol., 10 (2008) 612-621.
[38] E.J. Kennedy, L. Pillus, G. Ghosh, Pho5p and newly identified nucleotide pyrophosphatases/phosphodiesterases regulate extracellular nucleotide phosphate metabolism in Saccharomyces cerevisiae, Eukaryot. Cell, 4 (2005) 1892-1901.
[39] I. Alves-Pereira, J. Canales, A. Cabezas, P. Martin Cordero, M.J. Costas, J.C. Cameselle,

CDP-alcohol hydrolase, a very efficient activity of the 5′-nucleotidase/UDP-sugar hydrolase encoded by the ushA gene of Yersinia intermedia and Escherichia coli, J. Bacteriol., 190 (2008) 6153-6161.

[40] G. Kemmer, T.J. Reilly, J. Schmidt-Brauns, G.W. Zlotnik, B.A. Green, M.J. Fiske, M. Herbert, A. Kraiß, S. Schlör, A. Smith, NadN and e (P4) are essential for utilization of NAD and nicotinamide mononucleotide but not nicotinamide riboside in Haemophilus influenzae, J. Bacteriol., 183 (2001) 3974-3981.

[41] T. Elzainy, T. Ali, Nature and mode of action of NAD and ATP dephosphorylating enzyme from Aspergillus terreus DSM 826, Antonie Van Leeuwenhoek, 84 (2003) 255-260.

[42] R.A. Leigh, A.J. Pope, I.R. Jennings, D. Sanders, Kinetics of the vacuolar H+-pyrophosphatase: the roles of magnesium, pyrophosphate, and their complexes as substrates, activators, and inhibitors, Plant Physiol., 100 (1992) 1698-1705.

[43] A.A. BAYKOV, N.P. BAKULEVA, P.A. REA, Steady-state kinetics of substrate hydrolysis by vacuolar H+-pyrophosphatase: A simple three-state model, Eur. J. Biochem., 217 (1993) 755-762.