Purification and Characterization of Amidase from *Paracoccus* sp. SKG: Utilization of amidase inhibited whole cells for bioconversion of acrylonitrile to acrylamide

By

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I. Introduction

✓ Nitriles (organo-cyanide, RC≡N) are cyanide-substituted carboxylic acids produced naturally and synthetically.

✓ Naturally occurring nitriles are found in plants, bone oils, insects and microorganisms in low concentration complexed with other biomolecules.

✓ Synthetic nitriles have been extensively used as solvents, extractants, in the manufacture of pharmaceuticals and drug intermediates etc.

✓Important for synthesis of amines, amides, carboxylic acids, esters, aldehydes, ketones and heterocyclic compounds.

Acetonitrile

✓ **Acetonitrile** with mol formula CH_3CN, colourless liquid is
the simplest organic nitrile.

✓Widely used in the chemical industry as a starting material
for the synthesis of chemicals, pharmaceuticals, pesticides
and rubber products.

✓The most common use of this chemical as eluting medium in
HPLC.

✓The industrial production of acetonitrile was estimated to be
more than 40,000 tons per annum.

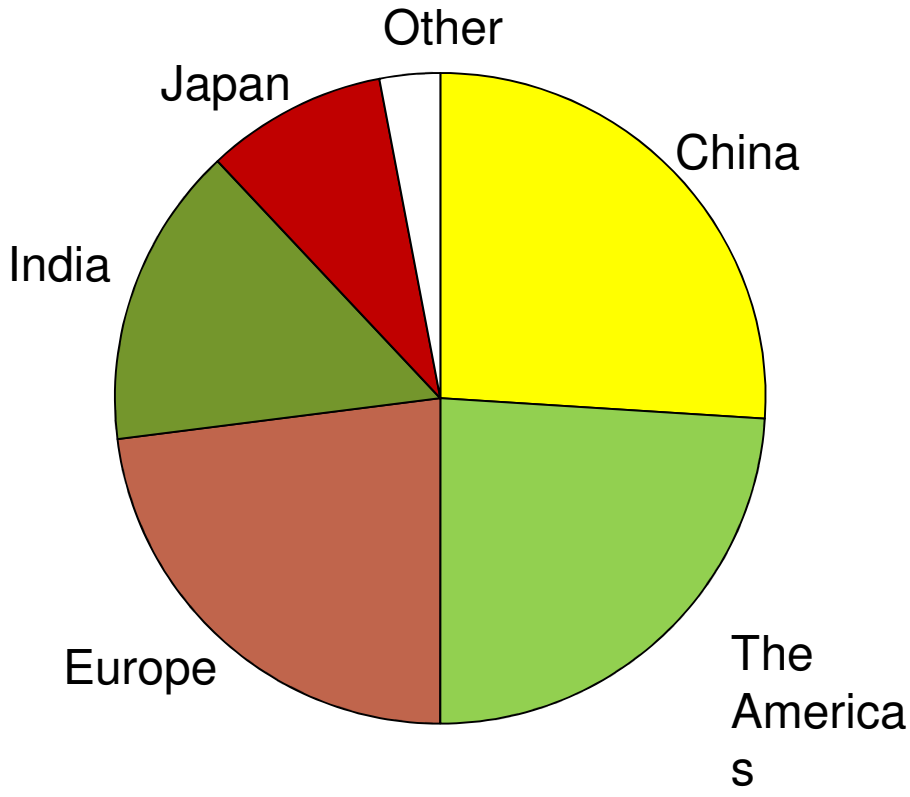


Fig 1. Worldwide consumption of acetonitrile in 2010

Toxicity of Nitriles

✓ The direct discharge of wastewater containing nitriles poses severe
health hazards.

✓Most nitriles are highly toxic and some are mutagenic and carcinogenic in nature.

✓Release of nitriles into water bodies results in letting cyanide, which persists in the soil or surface water causing severe environmental pollution.

❖ Therefore this study was undertaken for isolation, of bacteria degrading nitriles, purification and characterization of key enzyme. Further whole cells were used as biocatalyst for the production of amides and acids.

II. Isolation of bacteria capable of degrading aliphatic nitriles

- A bacterium capable of utilizing aliphatic nitrile as the sole carbon and nitrogen source was isolated from chemical waste samples.
- Employed selective enrichment culture technique.
- Culture was grown in MS medium devoid of carbon and nitrogen sources for nitrile degradation studies.
- Enrichment was carried out by transferring 5% inoculum to fresh minimal medium during which nitrile concentration was gradually increased.
- The purity of the culture was checked periodically by plating on LB agar plates.

Table 1: Morphological and cultural characteristics of strain SKG

Characteristics	Observation
Morphological characteristics	
Form	Small rods
Size	2.1µm x 0.51 µm
Gram stain	Gram negative
Motility	Motile
Flagella	Present
Endospore	Absent
Cultural characteristics	
Agar culture	Small and round colonies on acetonitrile plate
Agar slants	Smooth
Mc Conkey's agar	Colour less colonies
Pigmentation	Absent

Table 2: Physiological characteristics of strain SKG

Characteristic	Result
Growth on nutrient or L.B Broth	+++
Growth temperature	
5 ^{o}C	–
30 ^{o}C	+++
37 ^{o}C	++
45 ^{o}C	+
pH range for growth	6.5-9.0
Relation to oxygen	Aerobic

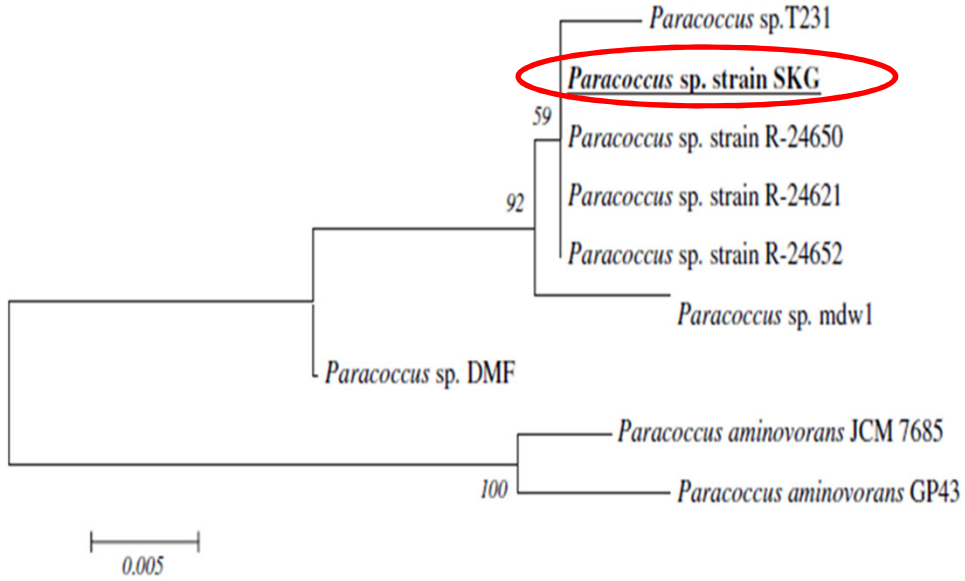


Fig 2. Phylogenetic tree of the strain SKG and related organisms based on 16S rDNA sequences.

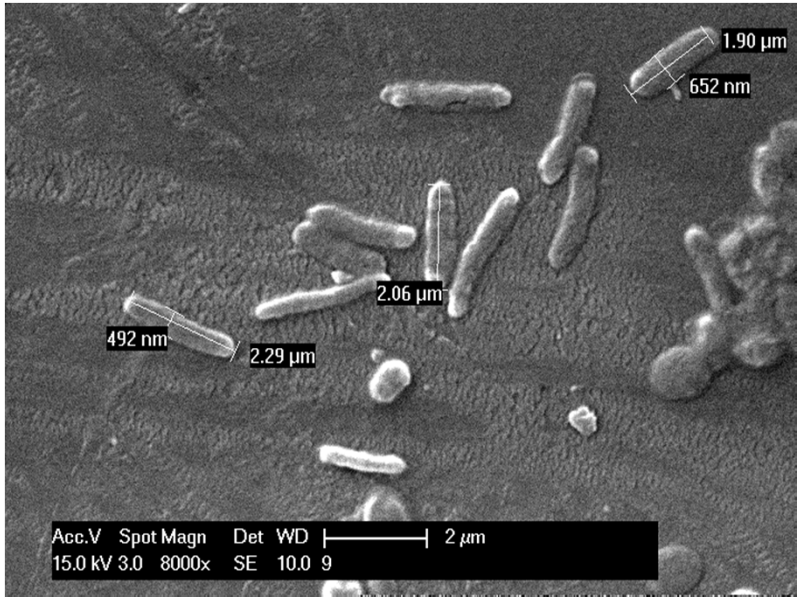
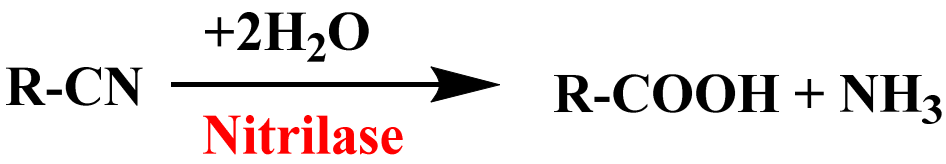


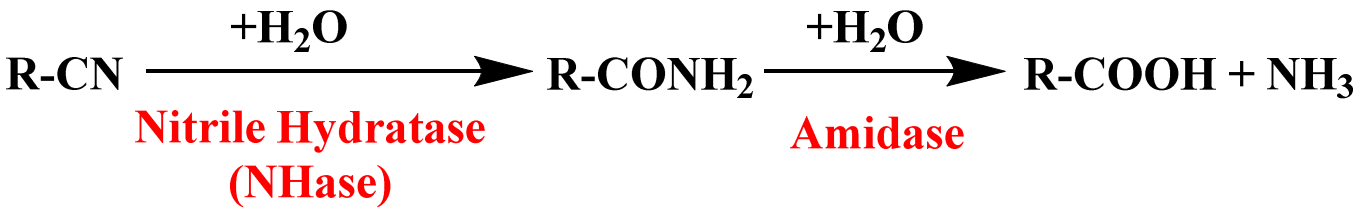
Fig 3. Scanning electron microscopic observation

III. Catabolic pathways for the degradation of nitriles

- In the first pathway, nitriles undergo direct hydrolysis to their carboxylic acids and ammonia by nitrilase (EC 3.5.5.1)



- In the second, nitrile hydratase (EC 4.2.1.84) catalyzes nitriles to amides which are then hydrolyzed to their respective carboxylic acids and ammonia by amidase (EC 3.5.1.4)



➢ Growth and utilization of acetonitrile by *Paracoccus* sp. SKG

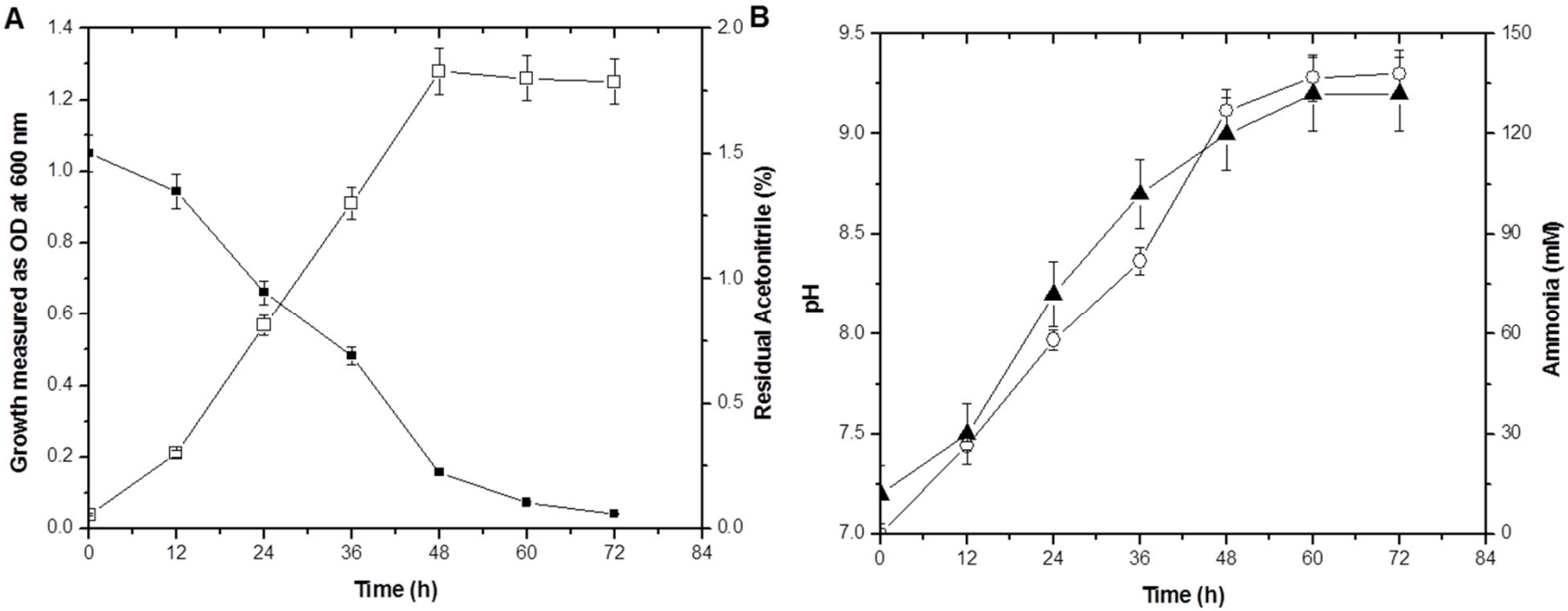


Fig 4. (A) Growth of SKG (□) and utilization of acetonitrile (■).
(B) pH of the medium (▲) and concentration of ammonia (○) released from acetonitrile degradation.

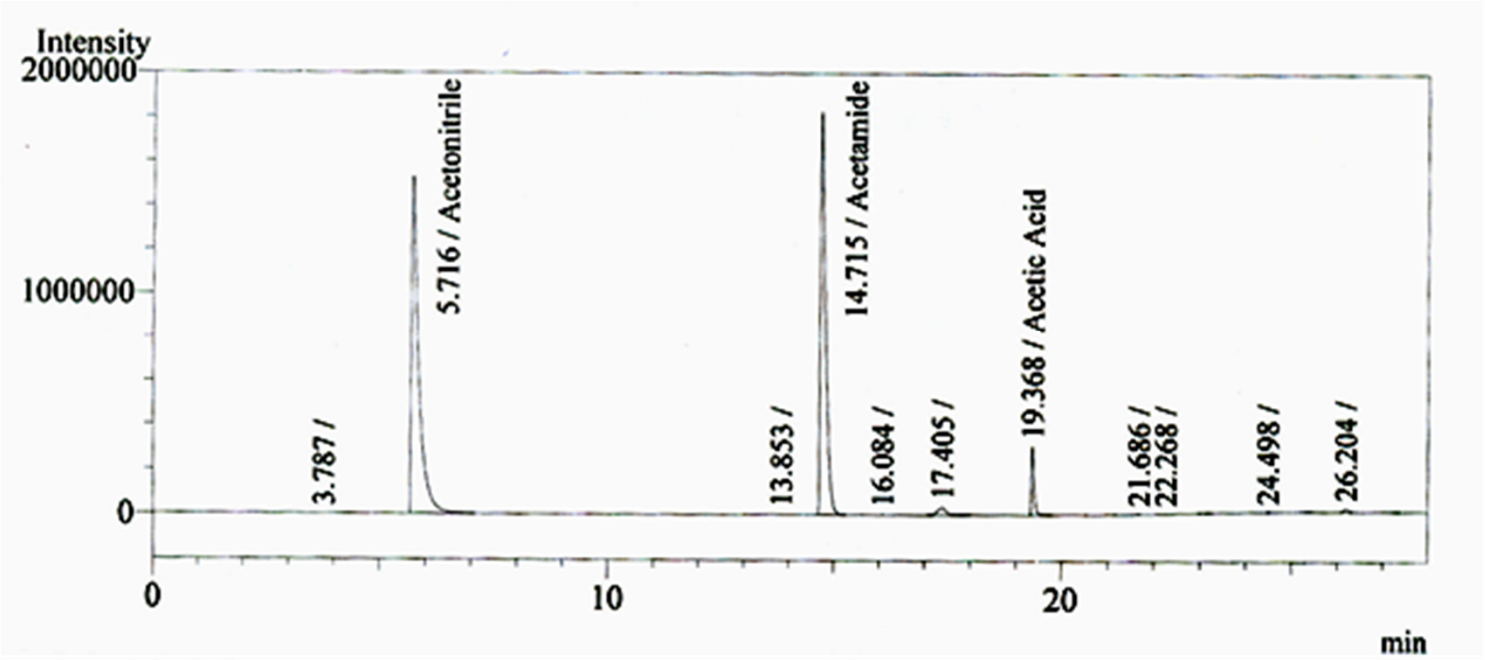


Fig 5. GC elution profile of metabolites of acetonitrile from spent medium after 48 h of incubation. (Retention time of acetonitrile: 5.71 min, acetamide: 14.71 min and acetic acid: 19.36 min).

Enzyme assays

- Nitrilase (ND)
- Nitrile hydratase (96 units)
- Amidase (274 units)

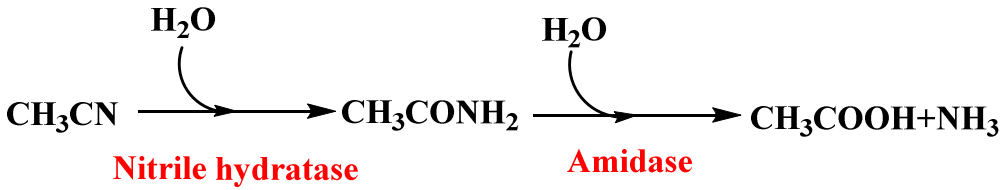


Fig 6. Degradation pathway of acetonitrile by *Paracoccus* sp. SKG.

IV. Purification and characterization of amidase

- ✓ Amidases or amidohydrolases (EC 3.5.1.4) are ubiquitous enzymes in the living world.
- ✓ Hydrolyze amides to the corresponding carboxylic acids and ammonia.
- ✓ Significant step in biotechnological applications in the production of enantiomerically pure intermediates.
- ✓ Amidases have extensive demand for industrial applications, such as production of optically pure compounds and waste water treatment.
- ✓ Purification and characterization of amidases will help to solve the problem of acrylic acid accumulation during acrylonitrile hydration to acrylamide.

Flow chart for purification of amidase

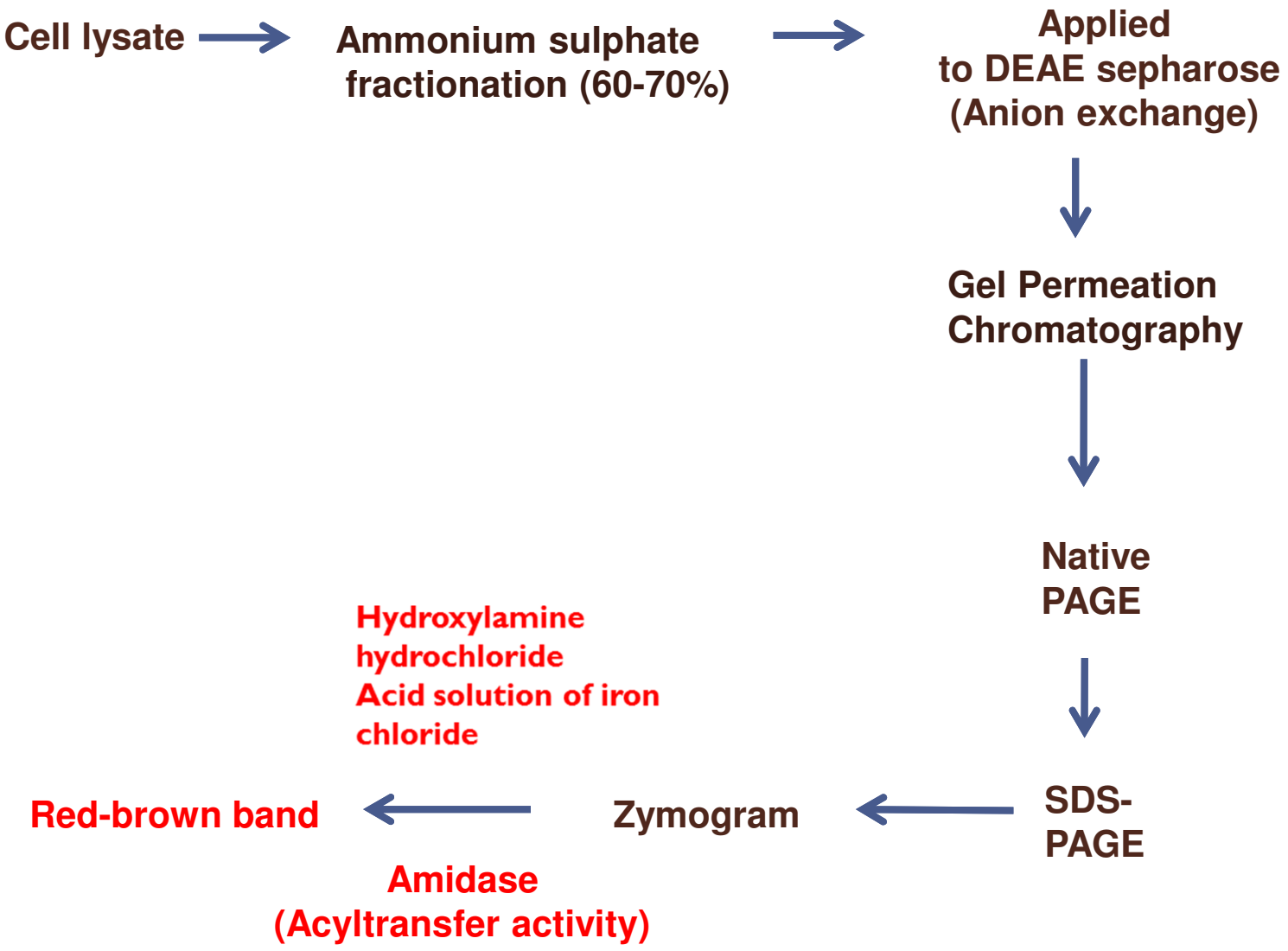


Table 3: Steps involved in the purification of amidase from *Paracoccus* sp. SKG.

Purification step	Total protein (mg)	Total activity (U*)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude enzyme	240	5160	21.5	1	100
Ammonium sulphate precipitation (40-60)	64	4290	67	3.11	83.1
DEAE fractionation	15	3705	247	11.4	71.8
Gel permeation fractionation	1.2	2400	960	44.6	46.5

expressed as units per mg of protein.

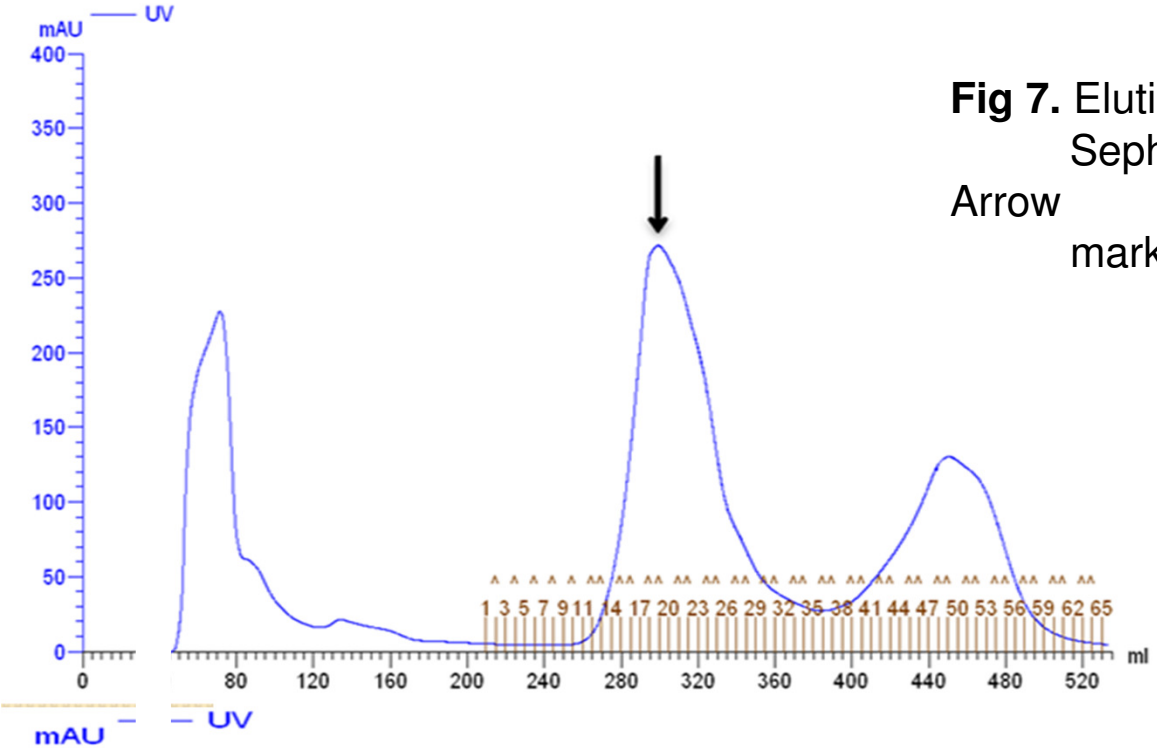


Fig 7. Elution profile of amidase from DEAE-Sepharose column chromatography. Arrow mark indicates the activity fraction.

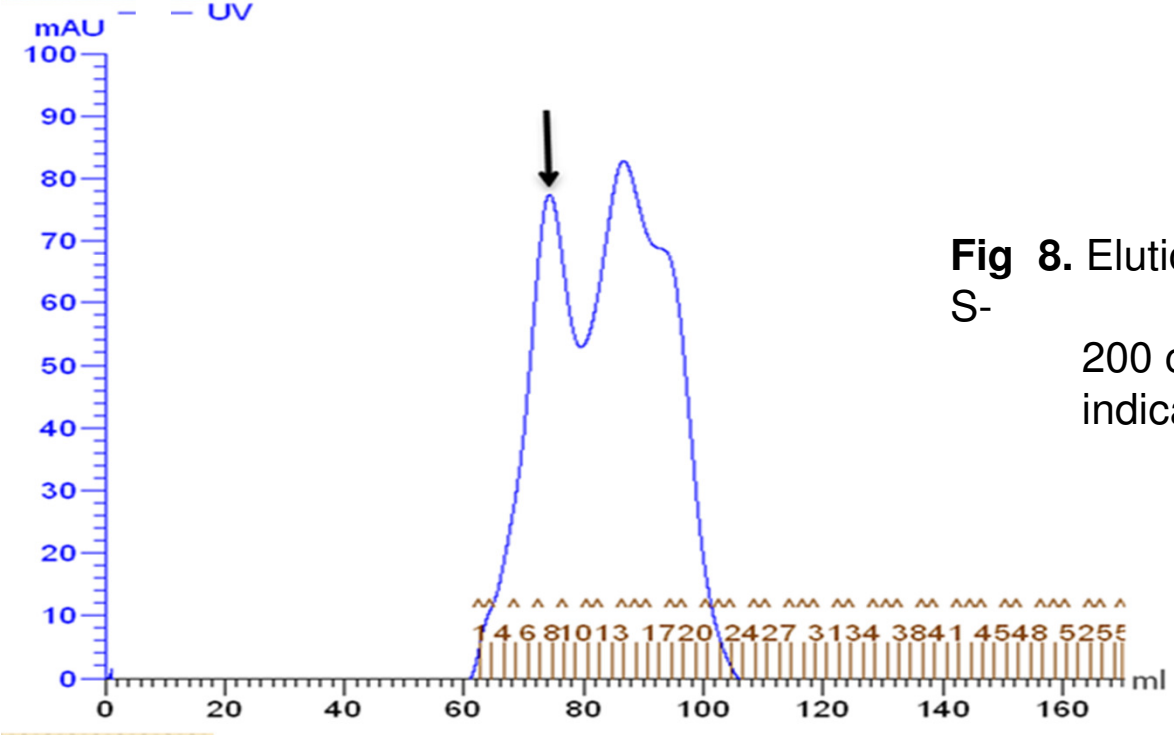
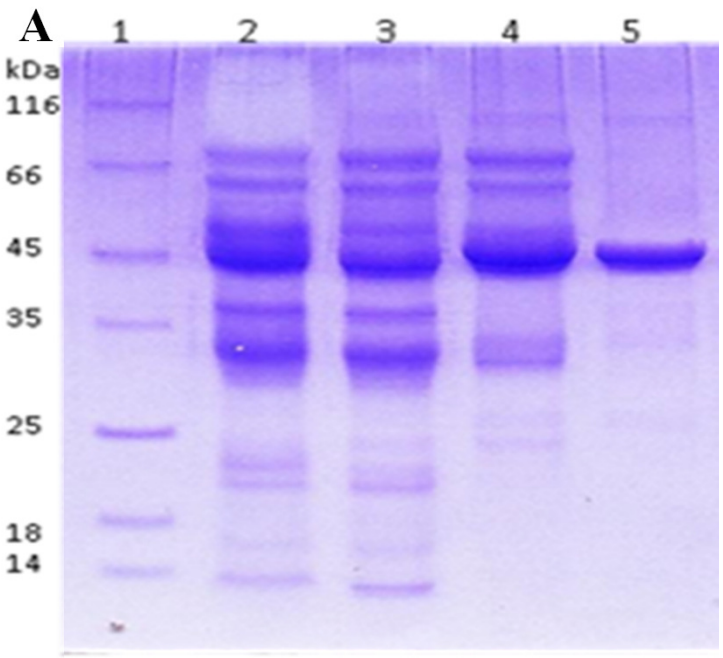


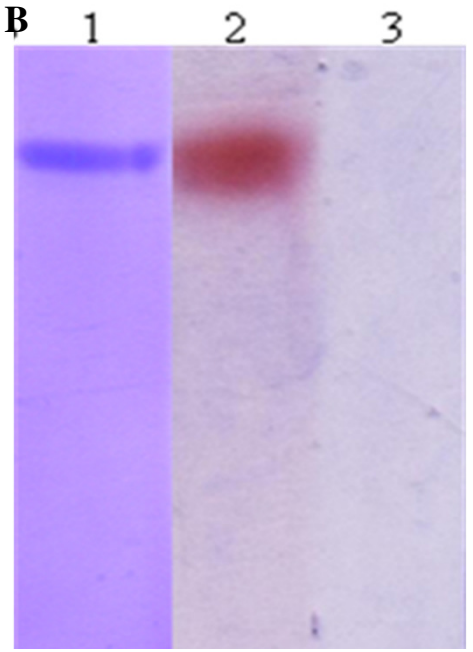
Fig 8. Elution profile of amidase from Sephacryl S-200 column chromatography. Arrow mark indicates the activity fraction.

Fig. 9. (A) SDS-PAGE analysis of samples containing amidase activity from different purification steps.



Lane 1. Protein molecular mass markers,
2. Crude extract,
3. Ammonium sulphate fraction,
4. DEAE Sepahrose fraction
5. Gel permeation fraction.

(B) Native-PAGE and zymogram activity of purified amidase (10 µg).



Lane 1. Purified amidase stained with coomassie brilliant blue
2. Amidase activity
3. Control for amidase activity (without substrate).

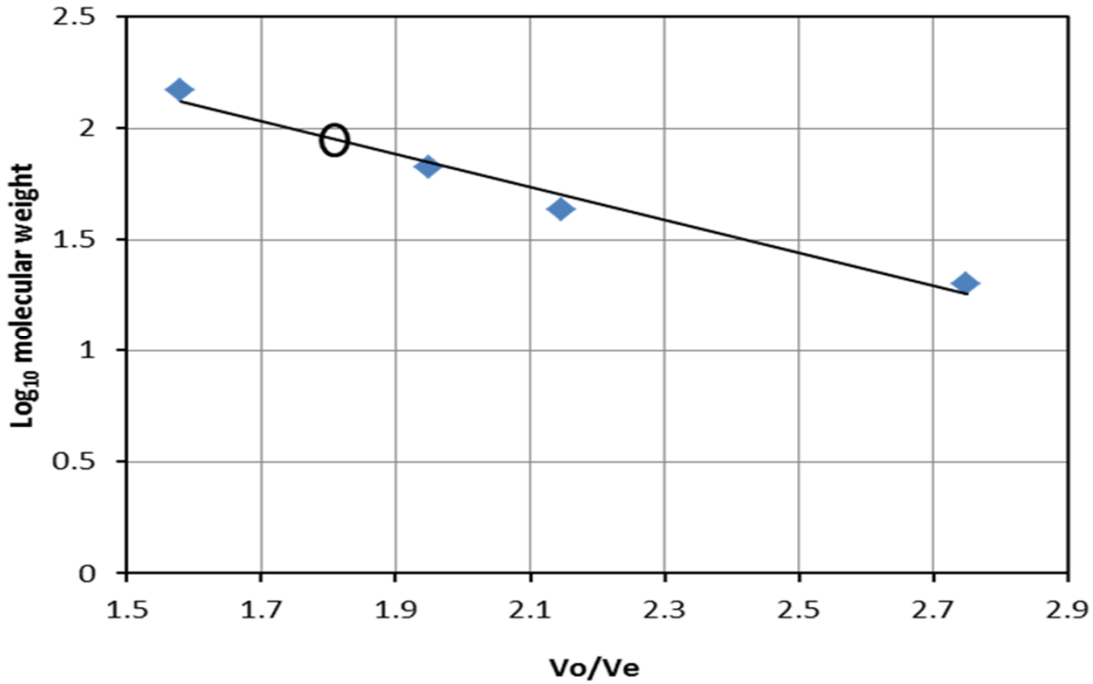


Fig 10. Molecular weight determination using gel filtration. Symbol (o) amidase and standard proteins are
alcohol dehydrogenase, albumin, ovalbumin and chymotrypsin.

✓ The amidase was purified to about 44.6 fold, with a recovery of 46.5%.

✓The purified enzyme migrated as a single band in SDS-PAGE with a molecular mass of 45 kDa.

✓Using gel filtration on a Sephacryl S-200 column, the molecular mass of the native protein was estimated to be 90 kDa.

✓Native enzyme consists of two identical subunits of 45kDa each.

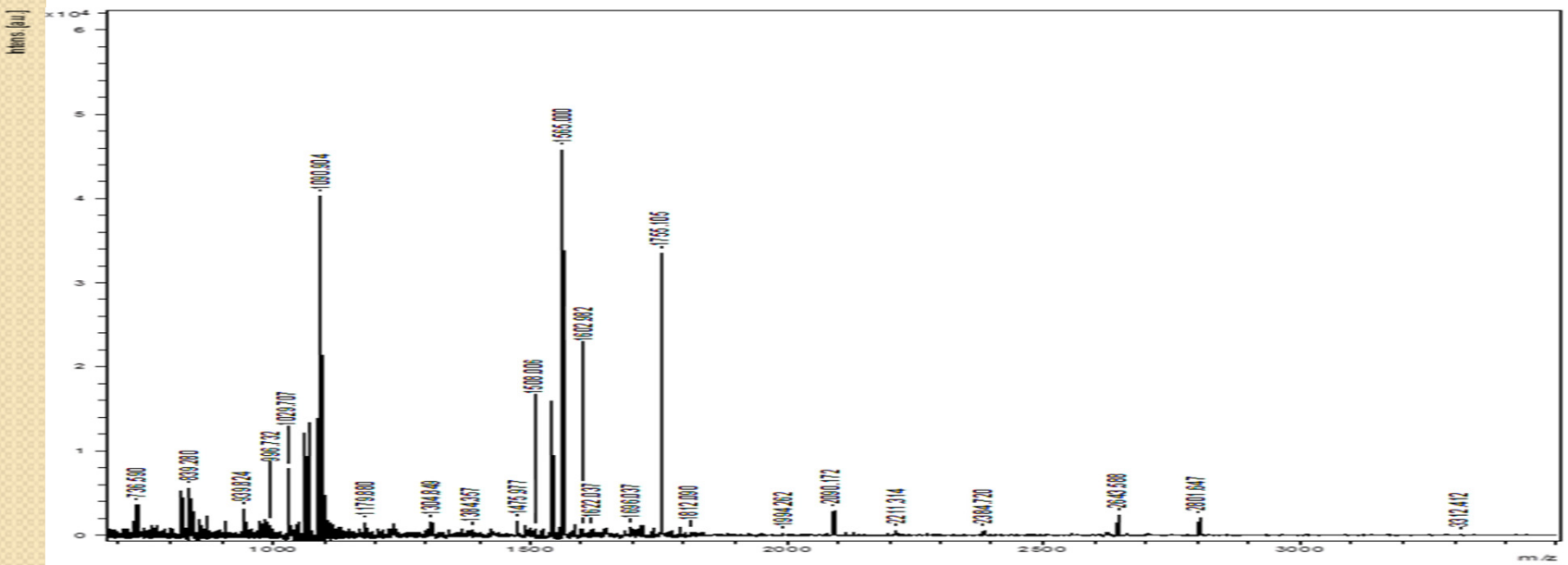


Fig. 11: MALDI-TOF mass spectrum of amidase from *Paracoccus* sp. SKG.

- The 45 kDa band excised from the gel was subjected to trypsin digestion.

- The peptide mass fragments (PMF) of purified amidase obtained from the MALDI-TOF were analyzed using a Mascot database search.

- Ten tryptic peptide fragments showed the highest identity with tryptic fragments of *Paracoccus denitrificans PD1222 amidase.*

- The identified *PMF* showed significant score and sequence

Table 4: Substrate spectrum of the amidase from *Paracoccus* sp. SKG. Amidase activity with acetamide as the substrate is considered as 100 %.

Substrate	Relative activity (%)
Acetamide	100.0
Propionamide	88.7
Acrylamide	61.8
Valeramide	52.3
Thiourea	18.0
Nicotinamide	11.4
Urea	03.6
Benzamide	00.0

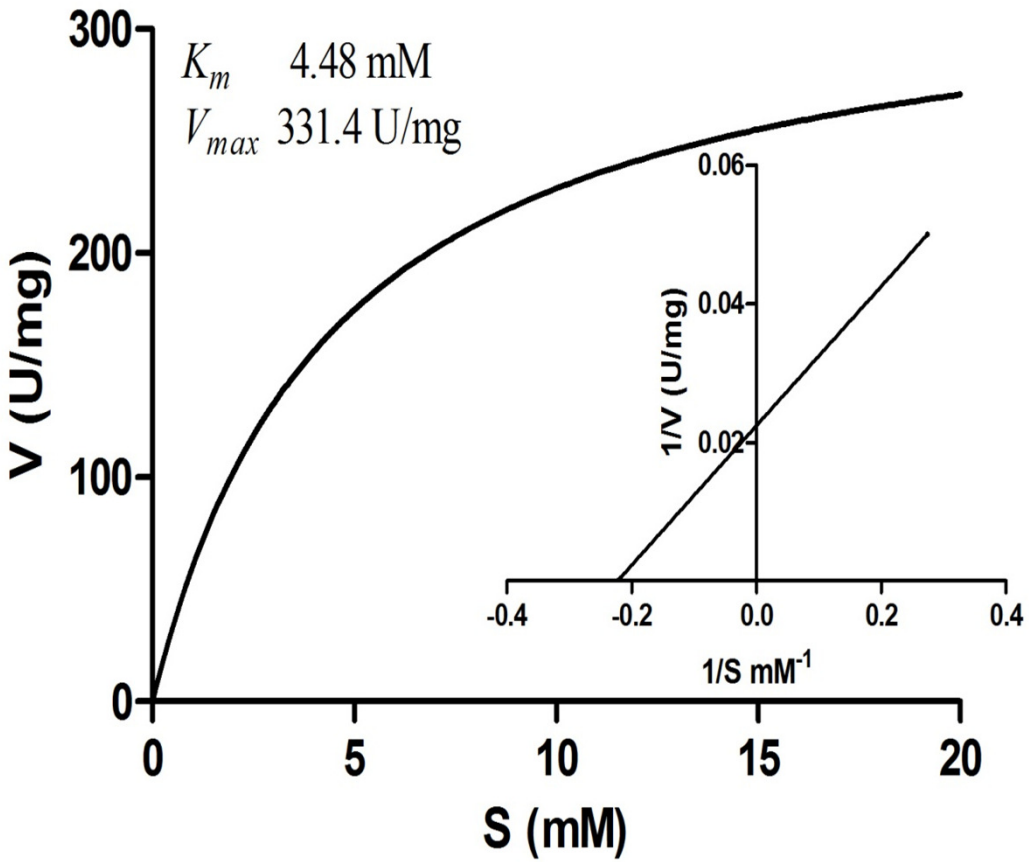


Fig 12. A plot of initial velocity [V] of Michaelis-Menten reaction versus the substrate concentration [S] with purified amidase showing hyperbolic curve with an acetamide substrate. The K_m and V_{max} for amidase are 4.48 mM and 331.4 U/mg of protein respectively. Inset: Lineweaver-Burk showing the K_m and V_{max} for amidase 4.4 mM and 331.40 U/mg of protein respectively.

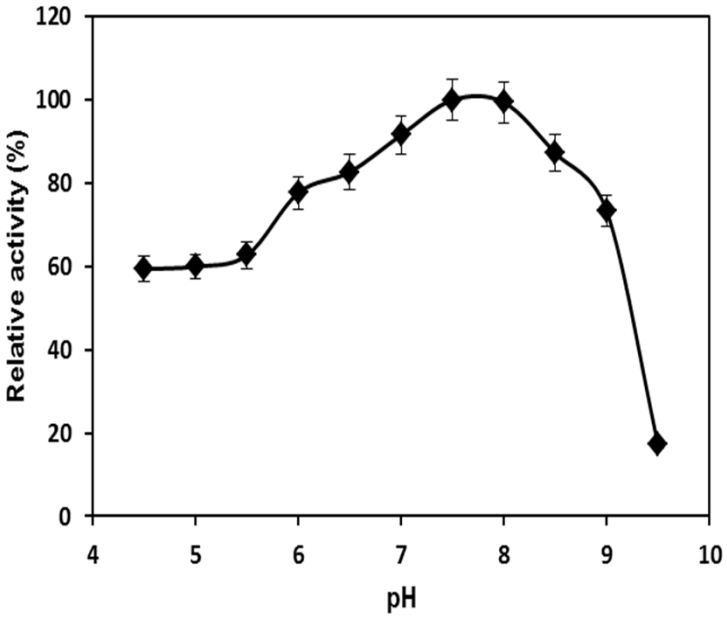


Fig 13. Effect of pH on amidase activity of *Paracoccus* sp. SKG. The amidase activity at pH 7.5 was considered as 100%.

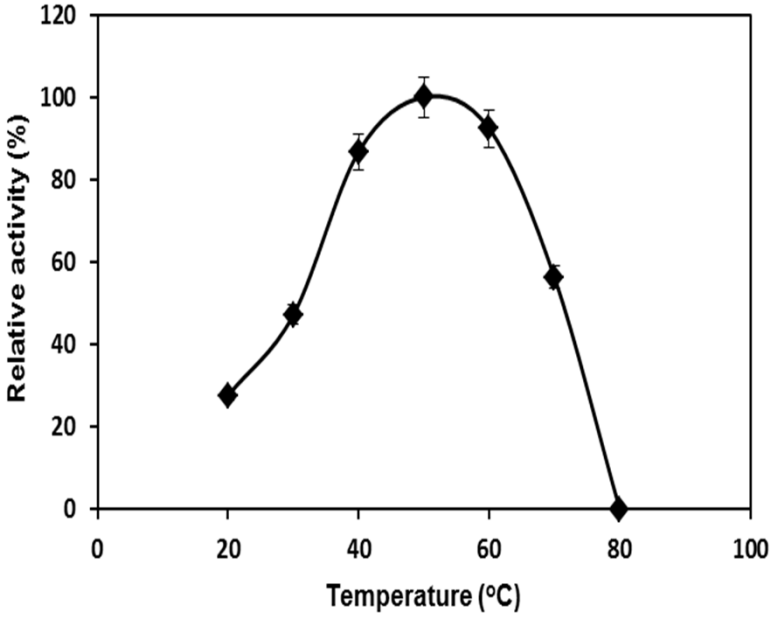


Fig 14. Effect of temperature on amidase activity of *Paracoccus* sp. SKG. The amidase activity at 50 °C was considered as 100%.

Table 5: Effect of various compounds on amidase activity.

Compound	Concentration (mM)	Relative activity (%)*
No addition	-	100.0
Mn^{2+}	1	146.1±0.32
Mg^{2+}	1	137.7±0.87
Ni^{2+}	1	117.2±0.64
Li^{2+}	1	114.5±0.34
Co^{2+}	1	108.3±0.51
Zn^{2+}	1	107.6±0.82
Ca^{2+}	1	101.2±0.21
Ba^{2+}	1	97.3±0.27
Fe^{3+}	1	94.0±0.43
Fe^{2+}	1	84.5±0.72
Cu^{2+}	1	00.0
DTT	1	120.5±0.68
EDTA	2	108.2±0.34
Triton X-100	1	101.5±0.18
SDS	0.1	82.0±0.35
Iodoacetate	1	46.8±0.79

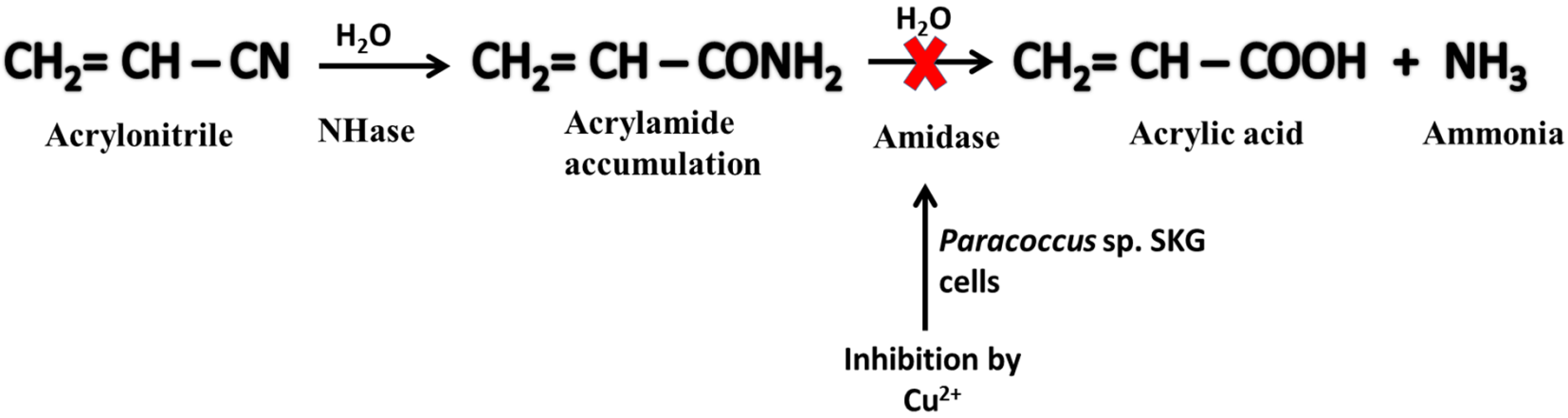
* Amidase assay without compounds is considered as 100% and data represent the mean ± SD, n = 3.

V. Bioconversion

- The conversion of one substance to another of higher industrial value by biological means.
- Bioconversion is becoming essential to the fine chemical industry in that their customers demand single isomer intermediates.
- In many cases, biocatalysis has replaced chemical catalysis because of
  - (i) Higher enantioselectivity and higher regioselectivity in aqueous solution
  - (ii) Does not require protection and deprotection of functional groups
  - (iii) Better stability
  - (iv) Operates under milder conditions
  - (v) Greater efficiency
  - (vi) Higher product yields

Biotransformation of Nitriles and Amides

- ***Paracoccus* sp. strain SKG: A potential biocatalyst for acrylamide production**



- Amidases are considered to be -SH proteins because they are inhibited by heavy metals such as mercury, copper and lead.
- The possible mechanism of the Cu^{2+} inhibition is due to heavy metals such as copper usually binds to the sulfhydryl group of cysteine in the active site of the enzyme leading to inactivation of the enzyme.

Acrylamide

- Acrylamide an important chemical used as coagulator, soil conditioner and stock additive for treatment in leather and textile industry.
- Acrylamide can be synthesized both chemically and enzymatically.
- Chemical method has some disadvantages, such as the rate of formation of by-product, acrylic acid in larger quantity than acrylamide and requiring high-energy input.
- Microbial bioconversion of acrylonitrile using whole cells having NHase has received much attention because of environment-friendly features.
- Acrylamide further transforms into acrylic acid through amidase catalysis , which is an undesirable feature.
- Amidase-inhibited whole cells of *Paracoccus* sp. SKG as biocatalyst for the production of acrylamide in a batch reaction.

❖ Preparation of Cu^{+2} treated resting cells of *Paracoccus* sp. SKG for use in bioconversion

Cells were grown in MM1 medium with 1.5% acetonitrile

↓

Log phase cells were harvested and washed with 50 mM PPB pH 7.2.

↓

Cells were pre-incubated with
1mM $CuSo_4$
10 min at room temperature

↓

Washed cells used for biotransformation

Table 6: Optimization of reaction conditions for bioconversion of acrylonitrile to acrylamide by using preincubated whole cells of *Paracoccus* sp. SKG.

Sl No.	Reaction condition	Tested range	Optimum conditions
1	$CuSO_4$ (mM)	1 - 10	1.0
2	50 mM potassium phosphate buffer (pH 6.0 – 8.5)	6.0 - 8.5	7.5
3	Temperature (^oC)	20-40	30
4	Cells concentration (mg dcw/ml)	0.5-10	2.0
5	Acrylonitrile (% v/v)	1 - 6	4.0

- One unit of NHase activity was defined as the amount of enzyme converting 1 µmol of acrylonitrile to acrylamide per min/mg of dcw.

Bioconversion of Acrylonitrile : Reaction Mixture

Buffer	Potassium phosphate buffer (50 mM)
pH	7.5
Substrate	Acrylonitrile , 4% (760 mM)
Biocatalyst	Preincubted cells of *Paracoccus* sp. SKG (2 mg dcm/ml)
Total reaction volume	100 ml

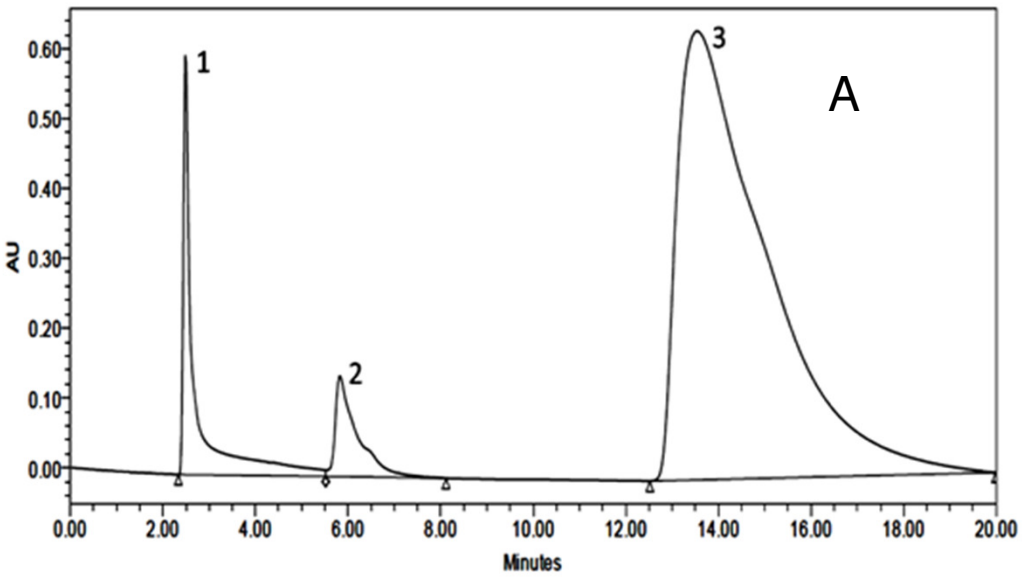
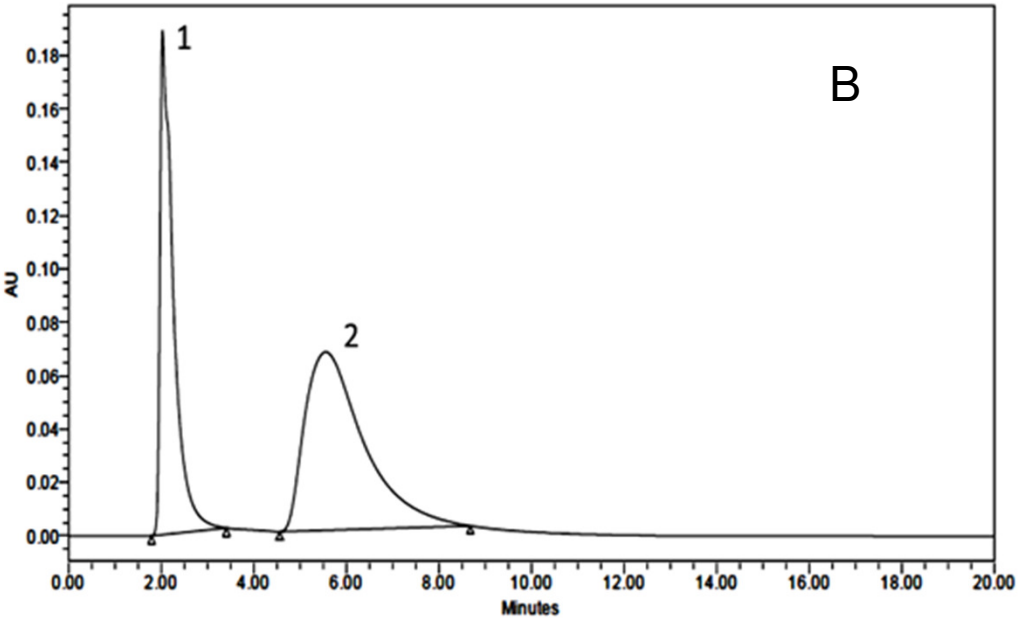


Fig.15. HPLC analysis of bioconversion of acrylonitrile to acrylamide by using whole cells of *Paracoccus* sp. SKG.

(A) Whole cells without preincubation with Cu^{2+}.



(B) Whole cells preincubated with Cu^{2+}.

Retention time of
(1) acrylamide: 2.3 min,
(2) acrylonitrile: 5.7 min,
(3) acrylic acid: 13.5 min.

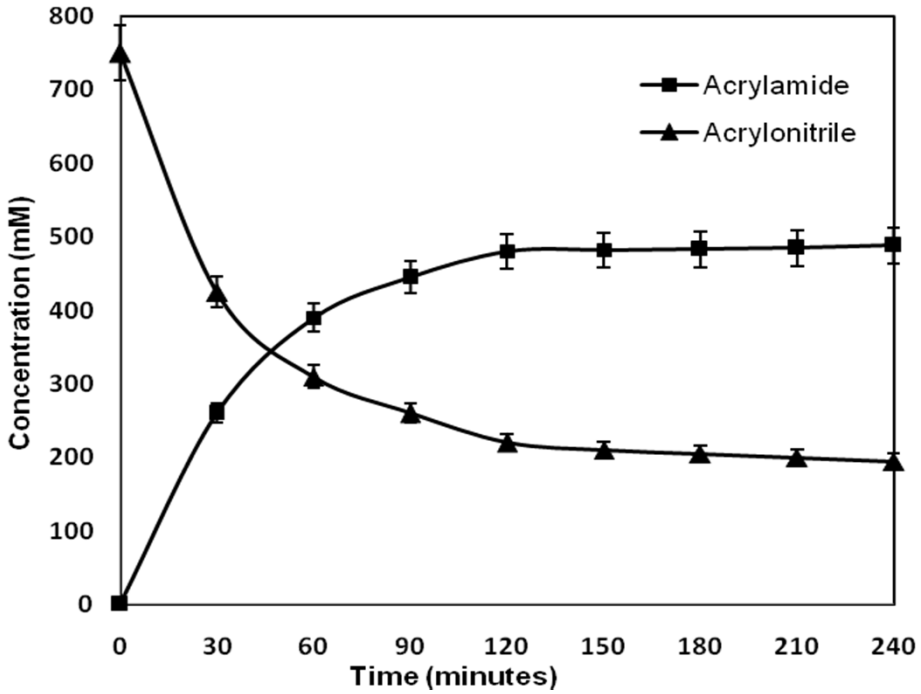


Fig 16. Time course conversion of acrylonitrile to acrylamide using preincubated whole cells of *Paracoccus* sp. SKG.

- Acrylamide recovered kept overnight at 0–4 °C for crystallization and dried at room temperature and weighed.
- The accumulation of acrylamide reached 2.7 g for 100 ml with 65%, i.e., 480 mM conversion.

Conclusions

- The isolated bacterial strain *Paracoccus* sp. SKG is able to degrade aliphatic nitriles.
- This strain has successfully removed 94 % of 1.5 % acetonitrile.
- Amidase from nitrile degrading *Paracoccus* sp. SKG was purified to homogenity and characterized.
- Further, the use of amidase-inhibited whole cells of *Paracoccus* sp. SKG was exploited as a biocatalyst for the production of acrylamide.
- The accumulation of acrylamide reached 27 g/L with 65% conversion of acrylonitrile in 2 h.

Acknowledgements

Research collaborators

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