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# Enzymatic and Anti-nutritive Degrading Activities of Mycelial Moulds Isolated from Amylolytic Starters of North East India

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Dried, oval to flat-shaped, ball-like traditionally made starters with variable sizes are prepared in North Eastern regions of India for fermentation of alcoholic beverages and drink from cereals. We screened some enzymatic activities such as amylase, cellulase, l-asparaginase, lipase, protease, xylanase and also antinutritive degrading enzymes such as laccase, phytase and tannase of 44 strains of mycelial moulds, which were previously isolated from different amylolytic starter cultures of North East India. *Aspergillus niger* NKM-8 showed maximum amylase activity of 27.67 U/ml. *A. flavus* SMM-1 showed high L asparaginase activity of 8.9 U/ml. *A. versicolour* APM-6 showed maximum protease activity of 54.6 U/ml. *Trametes hirsuta* MTM-12 showed the maximum cellulase activity of 15.6 U/ml. *Penicillium chrysogenum* SMM-16 showed xylanase activity of 7.8 U/ml. *T. hirsuta* MTM-12, *A. niger* NKM-8 and *A. niger* NKM-13 exhibited maximum laccase, phytase and tannase activities.

**Keywords:** Extracellular enzymes, Mycelial fungi, Mucor, Rhizopus, Starter cultures

## Introduction

Non-edible amylolytic and alcohol-producing solid cake or ball-like starter cultures are conventionally prepared by various ethnic communities of North East India (NEI) to produce alcoholic beverages from locally cultivated cereals, which are known as *chowan* in Tripura, *dawdim* in Mizoram, *hamei* in Manipur, *humao* and *xaj-pittha* in Assam, *khekhrii* in Nagaland, *marcha* in Sikkim and Darjeeling hills, *phab*, *phat* and *phut* in Arunachal Pradesh and *thiat* in Meghalaya.[1] Amylolytic and alcoholic-producing starters are dry, oval to flattened-shaped, solid, whitish, ball-like solid with variable sizes, which are prepared from rice or wheat as base materials.[2] Earlier we have isolated mycelial moulds from samples of *chowan, dawdim, hamei, humao, khekhrii, marcha*, *phut* and *thiat* and on the basis of morphological characters and ITS gene sequencing, 7 genera with 16 species were identified which included *Aspergillus sydowii*, *A. niger*, *A. flavus*, *A. versicolor*, *Bjerkandera adusta*, *Cladosporium parahalotolerans*, *Mucor circinelloides*, *M. indicus*, *Penicillium chrysogenum*, *P. citrinum*, *P. oxalicum*, *P. polonicum*, *Rhizopus delemar*, *R. microspores*, *R. oryzae*, and *Trametes hirsuta*.[3] The mycelial moulds present in amylolytic starters are known to produce various extracellular enzymes that degrade raw material during the fermentation.[4,5] Mycelial moulds are also known to produce some anti-nutritive degrading enzymes.[6] There is no report on enzymatic and anti-nutritive degrading activities of mycelial moulds present in traditionally-made amylolytic starter cultures of India. Hence, the present paper is aimed to screen the extracellular enzymes and anti-nutritive degrading enzymes of mycelial moulds, previously isolated from *chowan, dawdim, hamei, humao, khekhrii*, *marcha, phut* and *thiat.*

## Materials and Methods

### Sources of Mycelial Moulds

We used previously isolated and identified 44 strains of mycelial moulds from samples of *chowan, dawdim, hamei, humao, khekhrii*, *marcha*, *phut* and *thiat* (Table 1).(3) Sequences obtained were deposited at the GenBank-NCBI database under accession numbers: MK396469-MK396484, MK396486-MK396500, MK778442-MK778449, and MK796041-MK796045.(3)

### Screening of Extracellular Enzyme

#### *Amylase Activity*

The qualitative screening of 44 strains of mycelial moulds was performed for starch hydrolysis activity

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Table 1 — Screening of mycelial moulds-producing extracellular enzymes from amylolytic starters of North East India

| Starter | Isolate code | Mycelial moulds | Diameter in mm | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Amylase | | Cellulase | | L-Asparaginase | | Lipase | Protease | | Xylanase | | Laccase | | Phytase | | Tannase | |
| | | | Fungal colony | Zone of clearance | Fungal colony | Zone of clearance | Fungal colony | Zone of clearance | Zone of clearance | Fungal colony | Zone of clearance | Fungal colony | Zone of clearance | Fungal colony | Zone of clearance | Fungal colony | Zone of clearance | Fungal colony | Zone of clearance |
| *Marcha* | SMM-1 | *Aspergillus flavus* | 10 | 30 | — | — | 15 | 28 | 19 | 27 | 38 | 18 | 39 | — | — | — | — | 18 | 36 |
| | SMM-3 | *Mucor circinelloides* | — | — | — | — | 35 | 58 | - | — | — | — | — | — | — | — | — | — | — |
| | SMM-4 | *Rhizopus microsporus* | — | — | — | — | 34 | 50 | 24 | — | — | — | — | — | — | — | — | — | — |
| | SMM-10 | *Bjerkandera adusta* | — | — | — | — | 19 | 32 | — | — | — | 18 | 30 | — | — | — | — | — | — |
| | SMM-16 | *Penicillium chrysogenum* | 15 | 22 | — | — | 13 | 26 | — | 15 | 32 | 17 | 41 | — | — | 15 | 22 | — | — |
| | SMM-22 | *Penicillium polonicum* | — | — | 12 | 24 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | SMM-35 | *Penicillium chrysogenum* | 16 | 23 | — | — | 14 | 22 | — | 16 | 29 | 17 | 43 | — | — | 12 | 20 | — | — |
| *Thiat* | MTM-1 | *Mucor circinelloides* | — | — | — | — | 37 | 49 | — | — | — | — | — | — | — | — | — | — | — |
| | MTM-4 | *Rhizopus delemar* | — | — | — | — | 48 | 68 | — | — | — | — | — | — | — | — | — | — | — |
| | MTM-6 | *Penicillium chrysogenum* | 15 | 22 | — | — | 10 | 17 | — | 17 | 32 | 15 | 42 | — | — | 14 | 22 | — | — |
| | MTM-12 | *Trametes hirsuta* | — | — | 10 | 30 | — | — | — | — | — | 17 | 22 | 18 | 30 | — | — | 24 | 46 |
| | MTM-16 | *Bjerkandera adusta* | — | — | — | — | 14 | 36 | — | — | — | 15 | 29 | — | — | — | — | — | — |
| *Humao* | AEM-1 | *Penicillium citrinum* | 13 | 28 | — | — | 12 | 21 | 21 | — | — | 21 | 34 | — | — | 17 | 23 | — | — |
| | AEM-3 | *Rhizopus oryzae* | 30 | 38 | — | — | 28 | 40 | 38 | — | — | — | — | — | — | — | — | — | — |
| | AEM-4 | *Mucor circinelloides* | — | — | — | — | 36 | 56 | — | — | — | — | — | — | — | — | — | — | — |
| | AEM-8 | *Aspergillus sydowii* | 15 | 24 | 15 | 28 | 15 | 29 | — | 10 | 29 | — | — | 11 | 20 | 12 | 24 | — | — |
| | AXM-1 | *Aspergillus sydowii* | 16 | 25 | 16 | 27 | 14 | 27 | — | 8 | 25 | — | — | 12 | 23 | 11 | 22 | — | — |
| | AMM-3 | *Mucor indicus* | — | — | — | — | — | — | — | — | — | 28 | 36 | — | — | — | — | — | — |
| *Hamei* | MHM-1 | *Mucor circinelloides* | — | — | — | — | 34 | 46 | — | — | — | — | — | — | — | — | — | — | — |
| | MHM-15 | *Penicillium citrinum* | 9 | 22 | — | — | 11 | 21 | 19 | — | — | 19 | 43 | — | — | 14 | 21 | — | — |
| *Chowan* | TCM-1 | *Bjerkandera adusta* | — | — | — | — | 15 | 29 | — | — | — | 13 | 27 | — | — | — | — | — | — |
| | TCM-4 | *Mucor circinelloides* | — | — | — | — | 25 | 35 | — | — | — | — | — | — | — | — | — | — | — |
| | TCM-7 | *Rhizopus oryzae* | 31 | 39 | — | — | 22 | 39 | 36 | — | — | — | — | — | — | — | — | — | — |
| | TCM-9 | *Aspergillus sydowii* | 17 | 26 | 15 | 28 | 12 | 21 | — | 12 | 31 | — | — | 11 | 21 | 13 | 28 | — | — |
| | TCM-12 | *Penicillium chrysogenum* | 16 | 29 | — | — | 16 | 25 | — | 12 | 28 | 19 | 43 | — | — | 18 | 25 | — | — |
| *Phut* | APM-1 | *Aspergillus sydowii* | 12 | 25 | 16 | 26 | 17 | 26 | — | 11 | 25 | — | — | 13 | 22 | 11 | 19 | — | — |
| | APM-3 | *Mucor circinelloides* | — | — | — | — | 39 | 54 | — | — | — | — | — | — | — | — | — | — | — |
| | APM-6 | *Aspergillus versicolor* | 15 | 22 | — | — | 13 | 19 | — | 22 | 56 | — | — | — | — | — | — | — | — |
| | APM-7 | *Mucor indicus* | — | — | — | — | — | — | — | — | — | 22 | 35 | — | — | — | — | — | — |
| | APM-12 | *Rhizopus oryzae* | 32 | 39 | — | — | 20 | 36 | 39 | — | — | — | — | — | — | — | — | — | — |
| | APM-15 | *Aspergillus sydowii* | 14 | 26 | 14 | 24 | 16 | 25 | — | 10 | 18 | — | — | 14 | 23 | 12 | 20 | — | — |
| *Dawdim* | MDM-1 | *Mucor circinelloides* | — | — | — | — | 23 | 42 | — | — | — | — | — | — | — | — | — | — | — |
| | MDM-10 | *Bjerkandera adusta* | — | — | — | — | 12 | 26 | — | — | — | 16 | 21 | — | — | — | — | — | — |
| | MDM-11 | *Rhizopus microsporus* | — | — | — | — | 29 | 45 | 29 | — | — | — | — | — | — | — | — | — | — |
| | MDM-14 | *Mucor circinelloides* | — | — | — | — | 37 | 49 | — | — | — | — | — | — | — | — | — | — | — |
| | MDM-16 | *Bjerkandera adusta* | — | — | — | — | 13 | 27 | — | — | — | 19 | 32 | — | — | — | — | — | — |
| | MDM-18 | *Penicillium chrysogenum* | 16 | 23 | — | — | 15 | 23 | — | 19 | 34 | 18 | 32 | — | — | 16 | 27 | — | — |
| *Khekhrii* | NKM-1 | *Mucor circinelloides* | — | — | — | — | 34 | 42 | — | — | — | — | — | — | — | — | — | — | — |
| | NKM-6 | *Penicillium citrinum* | 10 | 21 | — | — | 12 | 18 | 18 | — | — | 24 | 46 | — | — | 15 | 29 | — | — |
| | NKM-7 | *Aspergillus flavus* | 11 | 38 | — | — | 13 | 25 | 22 | 28 | 51 | 17 | 32 | — | — | — | — | 17 | 32 |
| | NKM-8 | *Aspergillus niger* | 14 | 40 | — | — | — | — | — | — | — | — | — | — | — | 21 | 29 | 15 | 29 |
| | NKM-10 | *Penicillium oxalicum* | 15 | 24 | — | — | — | — | — | — | — | — | — | — | — | 11 | 24 | | — |
| | NKM-13 | *Aspergillus niger* | 12 | 42 | — | — | — | — | — | — | — | — | — | — | — | 18 | 28 | 17 | 30 |
| | NKM-15 | *Cladosporium parahalotolerans* | — | — | — | — | 8 | 18 | — | — | 40 | 14 | 29 | — | — | — | — | — | — |

—, no colony of fungi and no inhibition zone

as per the method of Carroll *et al*. (2017).[5] Amylase activity was performed using 3, 5- dinitrosalicylic acid as described by Omemu *et al*. (2015).[6]

***Cellulase Activity***

The qualitative and quantitative determination of cellulolytic activities were determined in the medium composed of Yeast Extract Peptone Agar with 0.5% Carboxy-methylcellulose (CMC).[7]

***L-asparaginase Activity***

Qualitative screening of L-asparaginase production was performed by plate assay method.[8] L-asparaginase activity was performed by submerged fermentation method.[9]

***Lipase Activity***

The phenol red media was used for qualitative screening of lipase enzyme and the lipase activity was

estimated according to method described by Lanka and Latha (2015).[10]

***Protease Activity***

The qualitative enzyme assay was used for the assessment of protease activity according to Patil *et al.* (2015).[11] Protease activity was measured by calculating one enzymatic unit representing the amount of enzyme that liberates 1µg of tyrosine under enzyme assay condition.[11]

***Xylanase Activity***

Screening for xylanase production was performed in medium containing 1% beechwood, 0.1% yeast extract and 1.6% agar[12], the xylanase activity was determined on the basis of liberation of reducing sugar from xylanase by 3, 5-dinitrosalicylic acid.[12]

**Screening of Antinutritive-Degrading Factors**

***Laccase Activity***

The mycelial moulds were screened for laccase enzyme by plate assay method using ABTS and guaiacol as a substrate and laccase activity was estimated following the method of Senthivelan *et al*. (2013).[13]

***Screening of Phytase***

Screening of phytase was performed in phytase screening medium (PSM) and the activity of phytase was estimated by determining the quantity of discharged inorganic phosphate.[14]

***Tannase Activity***

The qualitative determination of tannase and its activities was performed by the method of Cavalcanti *et al*. (2017).[15]

## Results and Discussion

Forty four strains of mycelial moulds, which were previously isolated and identified from amylolytic starters of 8 states located in NEI[2], were screened for enzyme production such as amylase, cellulase, L-asparaginase, lipase, protease and xylanase (Table 1). Out of 44 mycelial moulds, 22 strains showed amylolytic activities on starch agar by observing clear zone of starch hydrolysis (Fig. 1a). After primary screening, 22 mycelial moulds were assessed for their quantitative estimation under submerged fermentation conditions. *Aspergillus niger* NKM-8 isolated from *khekhrii* showed maximum amylase activity of 27.67 U/ml, followed by *A. niger* NKM-13 isolated from *khekhrii* 26.7 U/ml, and *A. flavus* SMM-1 isolated from *marcha* 22.06 U/ml, respectively (Fig. 2a). Various amylase producing microorganisms have been reported earlier.[16, 17]

Cellulase degrades the cellulose by cleaving the β–1, 4–glycosidic linkages.[18] Out of 44 tested moulds, only 7 strains showed cellulase activity (Fig. 1b). *T. hirsuta* (MTM-12) isolated from *thiat* showed highest cellulase activity of 15.6 U/ml (Fig. 2b), with high cellulolytic and saccharification properties.[19]

Out of 44 mycelial moulds, 37 strains showed L-asparaginase activities (Fig. 1c). *A. flavus* SMM-1 isolated from *marcha* and *A. flavus* NKM-7 isolated from *khekhrii* showed maximum L asparaginase activity of 8.9 U/ml and 7.28 U/ml, respectively. Only 10 fungal strains showed lipase production activity (Fig. 1d), out of which *R. oryzae* TCM-7 isolated from *chowan* showed highest value of lipase activity of 20.8 U/ml (Fig. 2d).

Out of 44, 13 strains showed proteolytic activities (Fig. 1e). *A. versicolour* APM-6 isolated from *phut* showed the maximum protease activity of 54.6 U/ml (Fig. 2e). *A. versicolour, A. flavus* and in *A. sydowii* are reported to produce good amount of protease.[20] Only 16 fungal strains showed xylanase activity (Fig. 1f), among which *Penicillium chrysogenum* SMM-16 isolated from *marcha* showed the higher

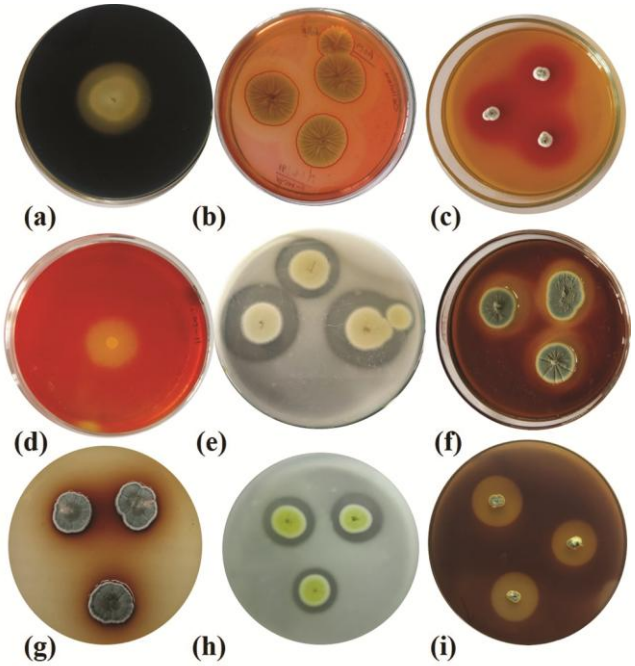


Fig. 1 — Plates showing clear zones (a) in 1% starch containing medium, (b) for cellulose activity, (c) for L-asparaginase activity, (d) for lipase activity, (e) for protease activity on gelatin agar plate, (f) for xylanase activity (g) for laccase activity, (h) for phytase activity, and (i) for tannase activity

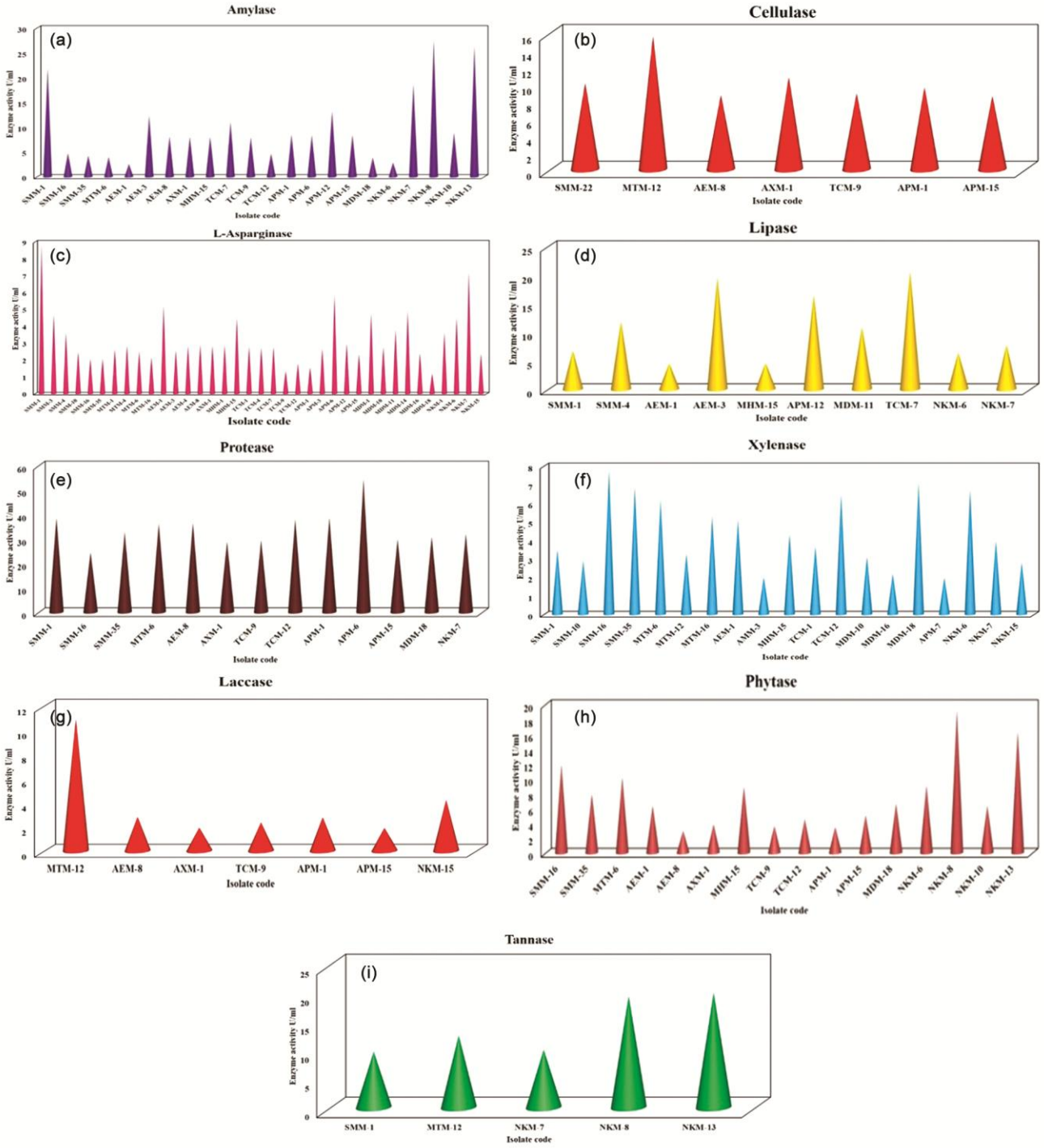


Fig. 2 — Screening of filamentous moulds for their enzyme activities (a) amylase, (b) cellulase, (c) asparaginase, (d) lipase, (e) protease (f) xylanase, (g) laccase, (h) phytase and (i) tannase (Fig. 2c and 2g are not cited in the running text)

xylanase activity of 7.8 U/ml followed by *P. chrysogenum* MDM-18 isolated from *dawdim* of 7.2 U/ml (Fig. 2f), *P. chrysogenum* produces high amount of xylanase.[21]

**Screening of Antinutritive-Degrading Factor**

Anti-nutritive factors such as tannins, phytic acid, protease and amylase inhibitors, present in cereals and legumes reduce nutrient bioavailability.[22] Filamentous

moulds present in fermented foods are able to produce the anti-nutritive degrading enzymes.[23] However, there is no such report of anti-nutritive degrading enzymes in dry amylolytic starters of Asia. Hence, we screened mycelial moulds, previously isolated from dry starter cultures of NEI, for their ability to degrade anti-nutritive factors using three standards enzymes viz. laccase, phytase and tannase (Fig. 1g, h and i) . *T. hirsuta* MTM-12 isolated from *thiat* showed 10.9 U/ml of laccase activity. *A. niger* (NKM-8) isolated from *khekhrii* showed highest phytase activity of 19.4 U/ml, followed by *P. chrysogenum* SMM-16 (12 U/ml) isolated from *marcha* and *P. chrysogenum* MTM-6 (10.2 U/ml) isolated from *thiat* 12.0 U/ml (Fig. 2h). *A. niger* NKM-13 isolated from *khekhrii* exhibited maximum tannase activity of 20.1 U/ml (Fig. 2i). Some mycelial moulds present in starter cultures of NEI have shown anti-nutritive degrading abilities, which may be useful to restore the nutrient bio-availability in alcoholic beverages, since these dry starters are used to produce the various traditional beverages in NEI.[24, 25]

## Conclusions

Some strains of mycelial moulds isolated from amylolytic starter cultures of NEI showed remarkable enzymatic activities as well as anti-nutritional degrading abilities. Hence, some of these mycelial moulds may also be exploited for industrial application for synthesis of some essential enzymes.

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