

Cold Active Amylases Producing Psychrotolerants Isolated from Nella Lake, Antarctica

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<http://dx.doi.org/10.13005/bbra/2603>

(Received: 03 March 2018; accepted: 20 March 2018)

Cold active amylase was investigated by bacteria and yeast isolates from the sediment core samples of Nella Lake, Larsemann Hills region, East Antarctica. Between potential yeast and bacteria isolates screened for amylases, best isolates were identified as *Rhodotorula* sp. Y-37 and *Arthrobacter alpinus* N16 by molecular technique. Amylase production capabilities of both the isolate subjected for optimization processes by using submerged fermentation technique with soluble starch as substrate. The results indicate that a supplement of 1% w/v glucose, 1% w/v yeast extract and 0.1% w/v KCl at pH 7.0 with 5% v/v inoculum enhances the amylase production by 5.72-fold using *Rhodotorula* sp. Y-37. In other hands, the activators are 1% w/v of galactose and peptone, 0.1% w/v KCl and 2.5% v/v inoculum at pH 7.0 enhances the amylase production by 3.74-fold using *Arthrobacter alpinus* N16. Cold-active amylase can be used in detergent, textile, food and beverage industries. Bio-degradation of starchy materials by cold active amylases can contribute in cleaning of environment at cold regions without harming the climate.

Keywords: Amylase, *Arthrobacter alpines*, Cold active, East Antarctica, Larsemann Hills, *Rhodotorula* sp.

An amylase is an enzyme that catalyses the hydrolysis of starch into sugars, are also regarded as glycoside hydrolases and act on α -1, 4-glycosidic bonds. It is of three types, i.e. α -amylases (EC 3.2.1.1), $\hat{\alpha}$ -amylase (EC 3.2.1.2) and $\tilde{\alpha}$ -amylase (EC 3.2.1.3). α -amylase acts up on starchy materials, and yields maltotriose, maltose and glucose as end product. Because it can act anywhere on the substrate, α -amylase tends to be faster-acting than $\hat{\alpha}$ -amylase having optimum pH is 6.7-7.0. While optimum pH for $\hat{\alpha}$ -amylase is 4.0-5.0 and $\tilde{\alpha}$ -amylase has the most acidic optimum pH 3.0.

Many researchers focused on thermophilic amylase, and cold-active amylase

very is less studied^{1,2,3}. Cold active amylase from bacteria like *Alteromonas* sp.⁴, *Nocardioopsis* sp.⁵, *Janthinobacterium* sp.⁶, *Arthrobacter* sp.⁷; yeasts like *Candida antarctica*⁸, *Cryptococcus flavus*⁹, *Rhodotorula glacialis* and *Mrakiagelida*¹⁰ and molds like *Aspergillus* sp.¹¹, *Penicillium* sp., *Alternaria* sp., *Absidia* sp. and *Fusarium* sp.^{12,13} were studied from various sources.

Cold-active amylases have immense biotechnological applications like additives in processed food industries and detergents, textile industries, waste-water treatment, bio-pulping, bio-remediations in cold climates and molecular biology applications^{14,15,16}. Thus, cold-active

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amylases are becoming promising enzymes and a world-wide choice for biotechnologists, microbiologists, biochemists and pharmacists. Cold active amylases function effectively at cold temperatures with high rates of catalysis in comparison to the amylases from their mesophilic or thermophilic counterparts, which shows little or almost no activity at a low temperature¹⁷. Cold active α -amylases save energy by overcoming the heating requirements, and minimize undesirable chemical reactions that could occur at high temperatures by removing contaminants¹⁸.

As cold-active enzymes have high catalytic activity at low temperatures with low thermo-stability at high temperatures, it is worthwhile to search for new cold active amylases with novel properties from different sources. There is no information available on enzymes from microorganisms isolated from Nella Lake, Antarctica, which may be an ideal habitat for cold adapted micro-organisms. Besides, primary screening optimization is necessary in the enzymatic study to make the yield commercially accepted¹⁹.

Therefore, in the present study psychrotolerant yeasts and bacterial strains have been isolated from the sediment core samples of Nella Lake and production of α -amylases are optimized through submerged fermentation technology. The investigation led to the identification of a high α -amylase producing isolates as *Rhodotorula* sp. Y-37 and *Arthrobacter alpinus* N16.

MATERIALS AND METHODS

Sample collection and isolation of bacteria and yeasts

Sediment core samples were collected from Lake Nella (76°22' S, 69°24' E), an ultra-oligotrophic lake located at an altitude of 15 m a.s.l. in the Broknes peninsula, Larsemann Hills region, East Antarctica. Core samples were cut off into small pieces from 0.5 cm to 90 cm, which were brought back to Polar Biology Lab, National Centre for Antarctic & Ocean Research with ice packs and kept at -20°C till investigation. Core samples were serially diluted by 10 fold dilution technique and spread over various media viz. Antarctic Biological Medium (ABM), Nutrient

Agar (NA), Zobell Marine Agar (ZMA), Potato Dextrose Agar (PDA) and their 1/10th strengths also used for isolating the oligotrophic bacteria and yeasts. Plates were incubated for 1-2 weeks at various temperatures viz. 1, 5, 15 and 22°C and cultures after proper visibility were streaked on the same agar plate for further purification using microscopic technique (Epi-fluorescence research microscope, BX51 Olympus, Japan). Purified isolates were stored at 1°C (Refrigerated incubator, MIR-554-PE, Panasonic) and glycerol stocks were also prepared in their respective broth with 20% glycerol (v/v) and kept at -20°C (Biomedical freezer, MDF-U537D, Sanyo).

Primary screening for cold active amylase

Amylase assay was done by taking soluble starch agar plates (g/L: beef extract, 3.0; peptone, 5.0; soluble starch, 2.0; agar, 20.0; pH 7.0) and incubating at 4, 15 and 22°C by spot inoculation method for 10 days¹². After incubation, Gram's iodine (Hi-media) was used for the zone of clearance.

Production and extraction of cold active amylase by submerged fermentation

Isolates were inoculated separately in Potato Dextrose Broth (PDB) and incubated at 15°C for 48 h at 150 rpm (Refrigerated incubator shaker, IS-971RF, Jeio tech, Korea). For production of amylase, freshly prepared inoculum was used. The production was done by using the modified mineral salt medium (%w/v: peptone, 0.6; KCl, 0.05; MgSO₄, 0.05; K₂HPO₄, 0.03; KH₂PO₄, 0.03; CaCl₂ · 2H₂O, 0.01; soluble starch, 0.1; pH 7.0) as recorded by Abe and co-workers with some modifications²⁰. The production medium was incubated at 15°C and 150 rpm after addition of 5% (v/v) inoculum to autoclaved medium for 120 h. Each day about 5 ml medium was pulling out aseptically and extraction was done by centrifugation at 10,000 rpm for 20 min at 4°C. The supernatant was regarded as crude amylase enzyme and subjected for assay.

Amylase assay

Amylase activity was measured spectrophotometrically (UV-Vis spectrophotometer, Specord S 205, Analytik Jena, AG Germany) using soluble starch (Hi-media) as a substrate²¹. Modifications were done in the reaction mixture incubated at 15°C for 20 min along with respective sample blanks. One unit of amylase activity is

defined as the quantity of enzyme that caused 0.01% reduction of blue color intensity of starch-iodine solution at 15°C in one min per ml at 690 nm.

Identification of potential isolates

Molecular identification of the potential isolates producing cold active amylase was done by using ITS-D1/D2 gene and 16s RNA gene sequencing based molecular technique for yeasts and bacteria, respectively. The isolates were outsourced to MTCC, Chandigarh, India for molecular identification. The gene sequences were used to carry out BLAST with the nr-database of GenBank database. The consensus sequence of sample was submitted at BankIt, GenBank, and NCBI for the accession numbers.

Partial characterization of potential isolates

The potential isolates were subjected for different biochemical tests (catalase, oxidase, MRVP, indole, citrate utilization, esculinase and phosphatase tests)²². Carbon utilization tests were carried out using HiCarbo™ kits (KB009, HiMedia, India) incubated for 2-7 days at 15°C. Besides, for physiological characterization, a loopful of culture was inoculated in flask containing 50 ml of broth and the flask were incubated at 5, 15, 22 and 35°C at 150 rpm in a shaking incubator. Growth was analyzed by spectrophotometer at 600 nm in the interval of 24 h. About 50 ml of broth with varied pH values (3, 5, 7, 9 and 11) and NaCl concentrations (0, 1, 5, 10 and 20% w/v) were tested for the growth of isolates at 15°C and 150 rpm in a shaking incubator. Furthermore, potential

isolates were screened for other hydrolytic enzymes like protease, lipase and cellulase.

Production optimization

Production optimization for cold active amylase was done by “one factor at-a-time” (OFAT) method. Factors investigated were incubation period (day 1-5), temperature (15 and 35°C), inoculum size (0.1-20% v/v), initial pH (5-12), nitrogen sources (1% w/v: yeast extract, beef extract, peptone, NaNO₃, KNO₃ and Ca(NO₃)₂·4H₂O) and carbon sources (1% w/v: glucose, maltose, fructose, lactose, galactose, sucrose and xylose), and minerals (NaCl, KCl, MgCl₂, MnCl₂, BaCl₂, HgCl₂ and CaCl₂). In each experiment, the optimized factor is replaced with the old one and a control devoid of additional factors was also incubated collaterally. All experiments were conducted in triplicates.

Statistical analysis

The data recorded during the investigation were subjected to significance testing by analyses of variance (ANOVA) using Microsoft excel 2007. Statistical significance was set at $P < 0.05$, and for high significance set at $P < 0.001$. Results were denoted as mean \pm standard error (SE) of triplicate experiments.

RESULTS

Screening and selection of potential amylase producers

All the bacterial and yeast isolates were investigated for amylase production at various

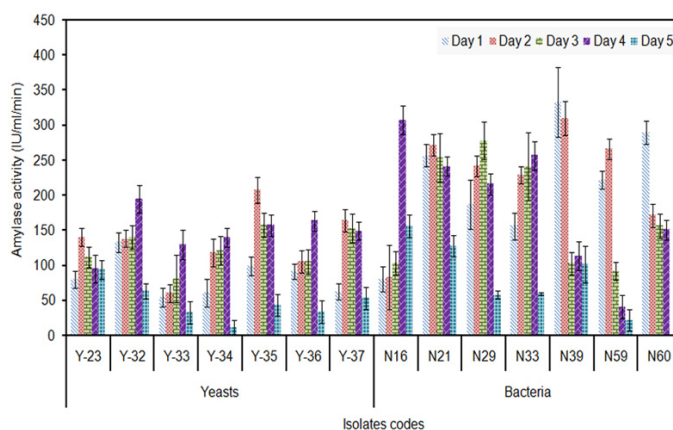


Fig. 1. Production of amylase by Nella Lake isolates using soluble starch at 15°C. The results are the means of 3 independent experiments, and the bars correspond to standard errors

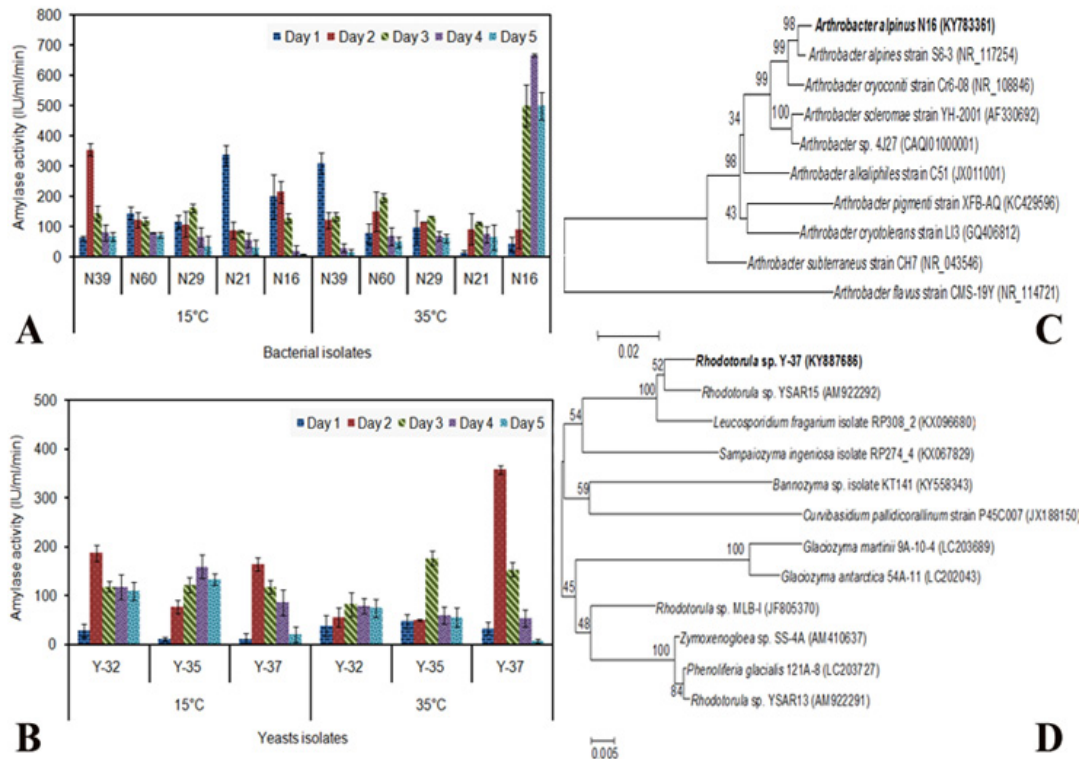


Fig. 2. Effects of incubation temperature on production of amylase by Nella Lake bacteria (A) and yeasts (B). Molecular phylogenetic analysis of N16 (C) and Y-37 (D), showing the position of strain N16 and Y-37 in relation to the other genera, GenBank accession numbers for the sequences are shown in parentheses; numbers at nodes denote bootstrap values based on 1000 replicates and evolutionary analyses were conducted in MEGA5

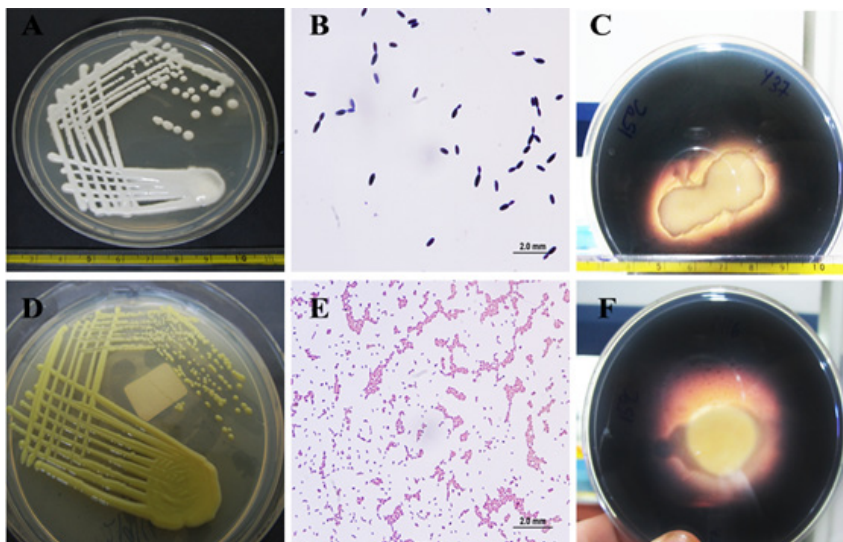


Fig. 3. A, Y-37 on potato dextrose agar. B, Micro-morphology of Y-37 observed under 60X. C, Amylase plate assay at 15°C by Y-37. D, N16 on nutrient agar. E, Micro-morphology of N16 observed under 60X. F, Amylase plate assay at 15°C by N16

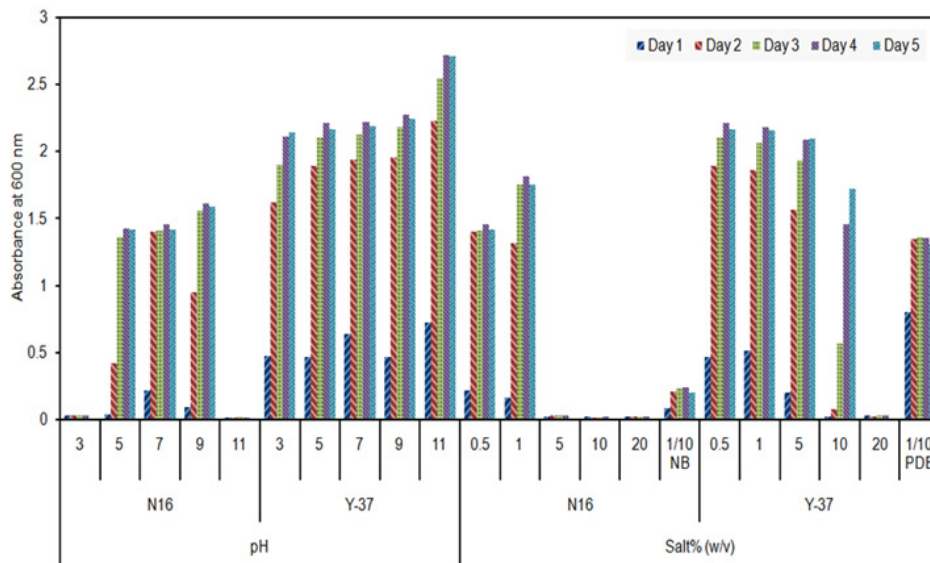


Fig. 4. Physiological analysis of *Arthrobacteralpinus* N16 and *Rhodotorulasp.* Y-37. The results are the means of 3 independent experiments. NB: Nutrient Broth; PDB: Potato Dextrose Broth

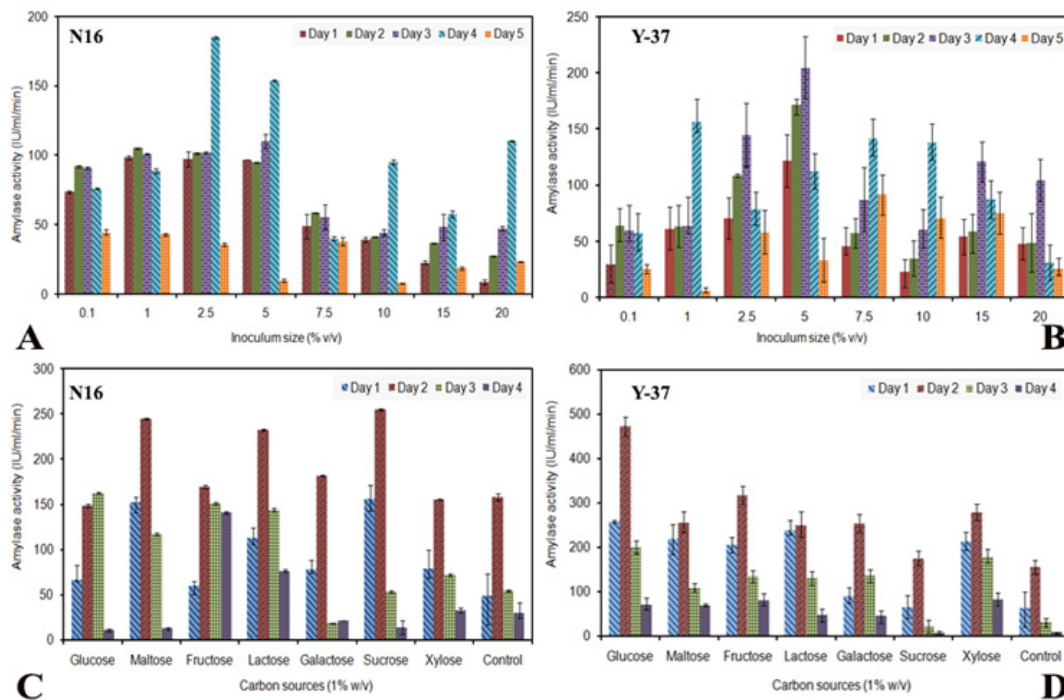


Fig. 5. Effects of inoculum size on production of amylase by *Arthrobacteralpinus* N16 (A) and *Rhodotorulasp.* Y-37 (B) and carbon sources on production of amylase by *Arthrobacteralpinus* N16 (C) and *Rhodotorulasp.* Y-37 (D). The results are the means of 3 independent experiments, and the bars correspond to standard errors

temperatures (4, 15 and 22°C) by plate assay method using soluble starch. Only 11.24% isolate were positive for amylase (data not shown). Then best seven from both yeasts (Y-23, Y32, Y-33, Y-34, Y-35, Y-36 and Y37) and bacteria (N16, N21, N29, N33, N39, N59 and N60) were selected for further quantification by submerged fermentation technique using soluble starch at 15°C for 5 days. Day-wise quantification was done and Fig. 1 revealed the maximum amylase produced by Y-35 and N39 among yeasts and bacteria, respectively. ANOVA revealed that there is a significant difference between the yeast and bacterial isolates producing cold active amylase at $P < 0.01$ while a high significant is seen i.e. $P < 0.001$ between the incubation period and amylase activity.

Effects of temperature on production of amylase

The best 3 isolates from yeasts (Y-32, Y-35 and Y-37) and 5 from bacteria (N16, N21, N29, N39 and N60) were investigated for amylase production at both 15°C and 35°C which revealed maximal production by the yeast isolate Y-37 i.e. 358.75 IU/ml/min at 35°C where as bacteria N16

showed maximum amylase 666.51 IU/ml/min at 35°C on 4th day of incubation (Fig. 2A, B). But as the amylase assay was conducted at 15°C the amylase can be regarded as cold active amylase. Further optimization was conducted by using Y-37 and N16 using various parameters. ANOVA reveals significant differences in amylase activities among the yeast isolates day wise at $P < 0.05$ while same significant is found in case of amylase activities among all the bacteria at different temperatures.

Identification and partial characterization of Y-37 and N16

From molecular identification, it was found that N16 is identified as *Arthrobacter alpinus* N16 (GenBank accession no. KY783361) and Y-37 as *Rhodotorula* sp. Y-37 (GenBank accession no. KY887686) and phylogenetic tree is represented in Fig. 2C, D. Macro- and micro- morphology of isolates N16 and Y-37 with amylase assay plates are depicted in Fig. 3A-F.

Rhodotorula sp. Y-37 can utilize various sugars as carbon sources viz. D-glucose, raffinose, sucrose, L-sorbose, D-gluconate, D-ribose,

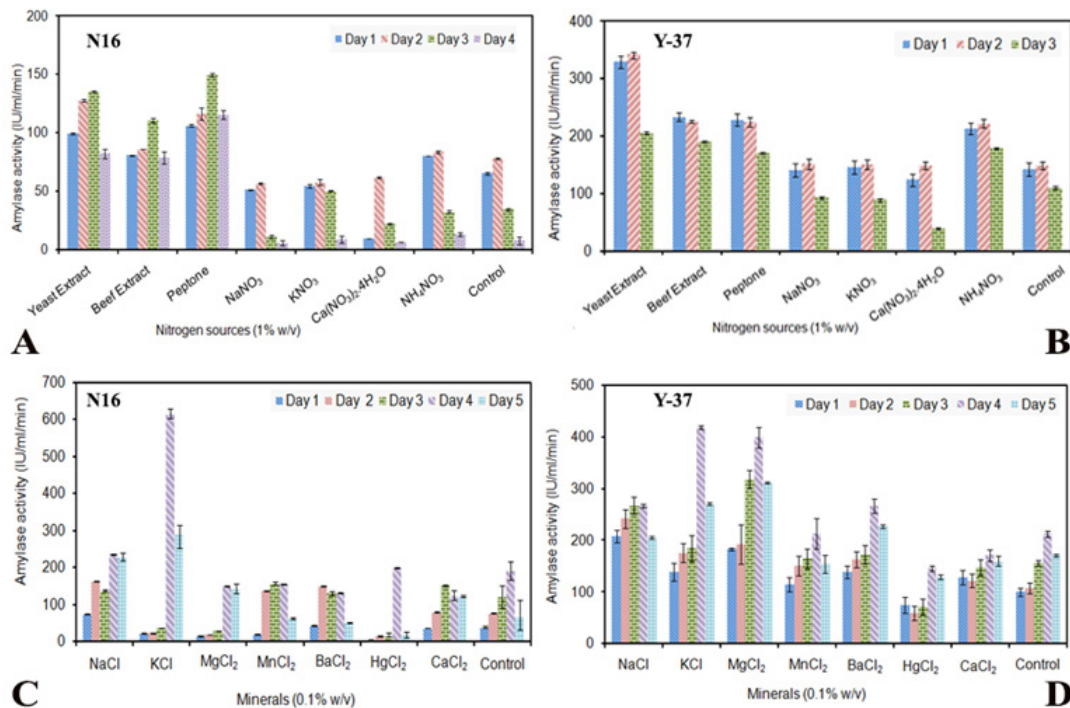


Fig. 6. Effects of nitrogen sources on production of amylase by *Arthrobacter alpinus* N16 (A) and *Rhodotorula* sp. Y-37 (B) and minerals on production of amylase by *Arthrobacter alpinus* N16 (C) and *Rhodotorula* sp. Y-37 (D). The results are the means of 3 independent experiments, and the bars correspond to standard errors

D-arabinose, lactose, glycerol, DL-lactate, myoinositol, D-xylose, maltose, cellobiose, melezitose, D-glucuronate, mannitol, D-galactose, rhamnose and L-arabinose. Between nitrogen sources, Y-37 utilizes nitrate, nitrite, lysine, tryptophan and creatinine. It is negative for growth at 1% acetic acid and 50% glucose. *Arthrobacteralpinus*N16 is an aerobic, Gram-positive rod actino-bacterium and was tested for many biochemical analyses. It is positive for catalase and negative for oxidase tests, MRVP, indole, citrate utilization, esculinase and phosphatase tests. Besides, it can utilize carbon sources viz. glycerol and inositol. Optimum temperature for the growth of both the isolate is 15°C, followed by 5°C. It was found that there is little growth at 35°C and almost no growth in case of 40°C. It denotes psychrotolerant nature of *Rhodotorula* sp. Y-37 and *Arthrobacteralpinus*N16. Both the isolate have a wide range of pH tolerance

from pH 3-11 while optimum is pH 9 and 11 by N16 and Y-37, respectively (Fig. 4). Salt tolerance tests revealed no growth found at 5%w/v by N16 while Y-37 can tolerate higher salt concentrations of 10% w/v. Y-37 is negative for cellulase while positive for protease and lipase while N16 can degrade substrate apart from starch, i.e. tributyrin (cold active lipase) and negative for protease and cellulase.

Effects of inoculum size on production of amylase

Various inoculum sizes ranging from 0.1-20 v/v were taken for optimal production of amylase by *Rhodotorula* sp. Y-37 and *Arthrobacteralpinus*N16 at 35°C and results are depicted in Fig. 5A, B. Maximum production was found by inoculum 5% v/v on 3rd day of incubation by Y-37 (204.35 IU/ml/min) while inoculum 2.5% v/v on 4th day of incubation was optimum for N16 (184.74 IU/ml/min). ANOVA reveals that there is a significant difference between the inoculum sizes

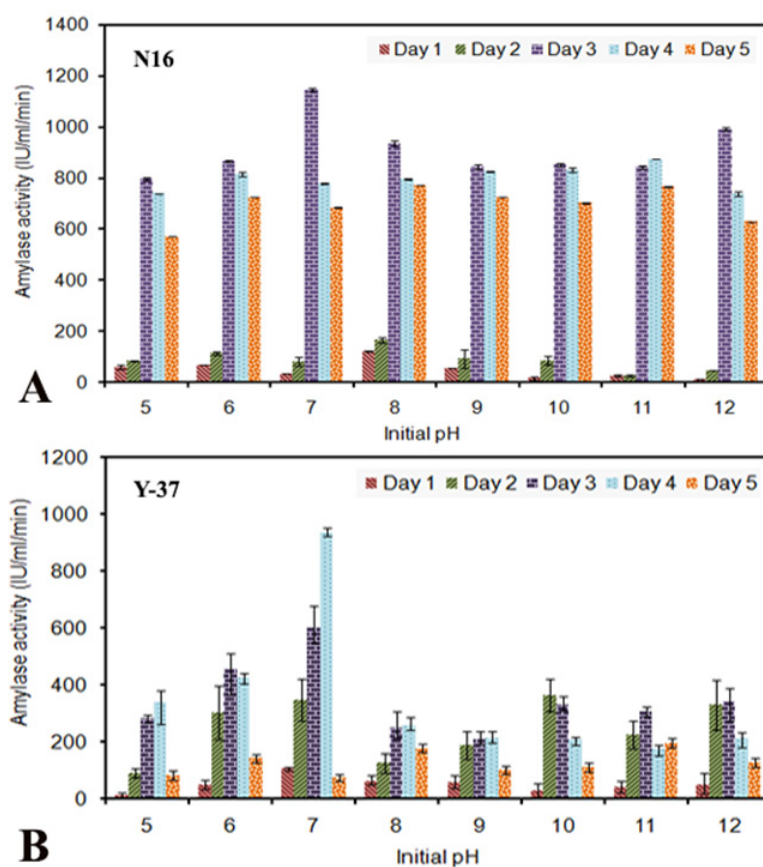


Fig. 7. Effects of initial pH on production of amylase by *Arthrobacteralpinus*N16 (A) and *Rhodotorula* sp. Y-37 (B). The results are the means of 3 independent experiments, and the bars correspond to standard errors

and cold active amylase activities at $P < 0.01$ along with the incubation period by both the isolate.

Effects of carbon sources on production of amylase

Among 1% w/v carbon sources were taken and added additionally into the production medium with a control having no additional carbon sources. Fig. 5C, D reveals all the carbon sources have induced effect on amylase production while glucose showed the maximum, i.e. 472.42 IU/ml/min by Y-37 and 1% sucrose was regarded as an optimum carbon source for N16. ANOVA reveals a high significant difference between the carbon sources investigated and cold active amylase activities at $P < 0.001$ along with the incubation period by both the isolate.

Effects of nitrogen sources on production of amylase

Various types of organic and in-organic nitrogen sources as mentioned earlier were taken and additionally put into the production medium with a control having no additional nitrogen sources (Fig. 6A, B). All the organic nitrogen sources have induced effect, and yeast extract showed the maximum, i.e. 342.33 IU/ml/min on 2nd day of incubation by Y-37. Same pattern was seen in case of N16 in less production due to inorganic nitrogen sources and optimum was found due to 1% peptone on 3rd day of incubation. ANOVA reveals a high significant difference between the nitrogen sources investigated and cold active amylase activity at $P < 0.001$ along with the incubation period by both Y-37 and N16.

Effects of minerals on production of amylase

Various types of mineral sources (0.1% w/v) were taken as mentioned earlier and added additionally into the production medium with a control having no additional mineral sources (Fig. 6C, D). All the minerals except $HgCl_2$, and $CaCl_2$ has induced effect on amylase production while KCl showed the maximum, i.e. 418.3 IU/ml/min on 4th day of incubation by *Rhodotorula* sp. Y-37. *Arthrobacter alpinus* N-16 showed maximum amylase production by 0.1% KCl, i.e. 615.51 IU/ml/min on 4th day of incubation. ANOVA reveals that there is a high significant difference between the minerals and cold active amylase activities along with the incubation period at $P < 0.001$ by Y-37 while at $P < 0.05$ by N16.

Effects of initial pH on production of amylase

Medium pH ranging from 5.0-12.0 was investigated, which revealed pH 7.0 as optimal for amylase production (936.74 IU/ml/min) on 4th day of incubation by *Rhodotorula* sp. Y-37 while *Arthrobacter alpinus* N16 produced 1147.51 IU/ml/min amylase on 3rd day of incubation (Fig. 7A, B). ANOVA reveals that there is a significant difference between the initial pH of the medium with that of cold active amylase at $P < 0.001$ along the incubation period by both Y-37 and N16.

DISCUSSION

Sediment cores from Nella Lake showed heavy loads of both bacterial and yeast strains at lower temperatures. Besides, only fewer strains were positive for amylase production at a cold temperature. Among them, two isolate one each from bacteria and yeasts was selected assuming both qualitative and quantitative methods. By molecular technique, isolates were identified as *Rhodotorula* sp. Y-37 and *Arthrobacter alpinus* N16. The isolate Y-37 showed closest similarity (98%) with *Rhodotorula* sp. YSAR15 (GenBank accession no. AM922292). YSAR15 was isolated from sediments of melt water stream of Arctic Midtre Lovénbreen glacier. This strain was positive for amylase more at 22°C as compared to 8°C²³. Isolate N-16 showed closest similarity (99%) with *Arthrobacter alpinus* strain S6-3 having GenBank accession no. NR_117254, a psychrophilic bacterium, which was isolated from alpine soil and cells exhibited a rod-coccus growth cycle and produced a yellow pigment and was also positive for starch degradation²⁴. There was a report of cold lipase produced by Antarctic strain *Rhodotorula* sp. Y-23 isolated from Nella Lake which also have ability to produce cold active amylase²⁵.

There are many reports on cold adapted amylase from bacteria^{17,22} and fungi^{26,12,13,27} from various sources. Carrasco and co-workers reported about yeasts isolated from King George Island, the sub Antarctic region in which *Cryptococcus* and *Rhodotorula* spp. showed amylase activity at a low temperature²⁸. Buzzini and Martini reported about amylase production by *Cryptococcus* and *Rhodotorula* spp. at 25°C isolated from Brazilian rain forest²⁹.

Rhodotorulamucilaginosa PT1, psychrotolerant yeast exhibited optimal amylase production at 25°C²⁶. In contrast, Hatha and co-workers reported about amylase produced neither by *Cryptococcus* nor *Rhodotorula* at 4°C or 20°C³⁰. The same pattern was also seen¹⁰. Present study contradicts with the results of Sahay (2015) who reported about a psychrotolerant *Rhodotorulamucilaginosa* BPT1 which showed maximal production (2.16 U/ml) of cold active amylase after 96 h with 1% (v/v) inoculum at 40°C incubation using 2% (w/v) soluble starch²⁷. However, there are many reports on cold active amylase production more at neutral pH by psychrotolerant *Rhodotorula mucilaginosa*^{26,27}.

Temperature is the most vital factor that not only affects the growth of microorganism but also meant for the thermo stability nature of enzymes. Present study reported maximum production was found at 35°C as compared with 15°C which denotes the psychrotolerant nature of both the isolates and also denotes the stability of amylases produced at that condition. As the assay was done at 15°C with 1% soluble starch, therefore it can be regarded as cold tolerant amylase. Enzymes from psychrotolerants are much important than the enzymes from psychrophilic and mesophilic counterparts, which may be due to a wider range of temperature tolerance. Besides, there are many reports of getting amylase from psychrotolerant isolates at a higher temperature than colder²⁶⁻²⁷. Cold tolerant amylase can be used in textile industries for desizing of grey fabrics, and low temperature saves energy consumptions effectively. Another application is fermentation of starch to ethanol, for which in general much higher temperature is needed with heavy applications of amylase. However, by introduction of cold active amylase, it minimizes energy consumptions with minimal enzyme concentrations for optimal production of ethanol from starchy materials¹⁷. To control the freezing point of frozen foods maltotetraose syrup was used, which is produced by starch with amylase³¹. Smita and co-workers reported on *Lactobacillus plantarum* producing cold active amylase best at 35°C on 36 h of incubation³². Munaganti and co-workers³³ reported about α -amylase produced by *Arthrobacter kerguelensis* VL-RK_09 which showed maximum amylase activity induced by

yeast extract at pH 7.0 on 4th day of incubation at 35°C whereas maximum cold active α -amylase production at 4°C was reported from Antarctic psychrophile *Alteromonas haloplanktis*³.

Inoculum size is a most important factor for initialization of enzyme production. Lower or much higher concentrations of inoculum size is destructive for enzymes, as an increase in inoculum size leads to increase in the duration of the initial lag phase³⁴. In contrast, lower inoculum size needs more time to grow and have slower log rate leads to lower production of enzymes³⁵.

Amylase is an inducible enzyme and is generally induced in the presence of starch or its hydrolytic product, maltose and organic nitrogen sources, have been preferred for the production of α -amylase³⁶⁻³⁸. Similar result of getting organic nitrogen sources for better amylase production was found in this present study. Aiyer (2004) reported about different carbon and nitrogen sources impact on production of amylase by *Bacillus licheniformis* SPT 27 who reported about more production using organic nitrogen sources than inorganic sources and optimum was achieved by peptone³⁹. Amylase is regarded as metallo-enzyme, therefore production is induced by the introduction of metal ions. Sivaramakrishnan and co-workers reported about α -amylase by *Bacillus cereus* MTCC 1305, revealed glucose showed enhanced enzyme production⁴⁰. Roohi and co-workers reported cold active amylase produced by *Microbacterium foliorum* GA2 isolated from Gangotri glacier, Western Himalaya, India³⁸. They reported about media factors for optimal production of amylase and reported 1% w/v yeast extract, and metal ion Mg²⁺ regarded as good producers whereas Ca²⁺ and Hg²⁺ showed inhibitory effects on amylase production. It corroborates with our present study.

Initial pH of the production medium affects the growth rate of microorganisms, leads to enzyme production. Inappropriate pH in the production medium alters the 3-D shape of the enzyme which in turn reform protein recognition and the enzyme might become inactive⁴¹. Present study reveals a neutral pH for both the isolate Y-37 and N16. Besides, N16 showed a wide range of pH tolerance from pH 5-12 indicating the amylase as novel enzyme, which can be used in clothing and dishwasher detergents to dissolve starches from

fabrics and dishes, respectively. Cold active and alkaline amylases protect the colour of delicate clothes. There are many reports of getting more amylases at pH 7.0^{32,33,42,43} which endorse our results.

CONCLUSIONS

From this study, it can be concluded that Nella Lake isolates have the potentiality to produce cold active amylase and various production parameters were investigated for the hyper amylase production. It successfully produced 5.72 fold increase in amylase production using *Rhodotorula* sp. Y-37 and 3.74 fold using *Arthrobacter alpinus* N16 from un-optimized conditions. The studied cold and alkaline active amylase can be used in various industries like food and beverages, detergents, textiles, paper and bakery industries. Besides, this is the first report on the microbiological study from Nella Lake, Antarctica as per best of our knowledge, which may lead to get isolates having novel metabolites for the usefulness of mankind. Furthermore, biodegradation of starchy materials at cold regions by the application of cold active amylases make it a harmless application as enzymes are non-toxic in nature, which contribute a greater role in cleaning of environment.

ACKNOWLEDGEMENTS

This study was financed by institutional project "Microbial Diversity of Antarctica" at National Centre for Antarctic & Ocean Research, Vasco-da-Gama, Goa, India. Authors are grateful to the Director, NCAOR, Goa, for facilities and Ms. Simantini Naik for technical help. Authors want to thank Dr. Alok Kumar Srivastava, Principle Scientist, Plant Pathology, ICAR-NBAIM, Kushmaur, U.P. and Dr. G.S. Prasad, Chief Scientist, Institute of Microbial Technology, Chandigarh for molecular identification of isolates.

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