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Maximization of Intracellular Lipase Production in a Lipase-Overproducing Mutant Derivative of *Rhizopus oligosporus* DGM 31: A Kinetic Study

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Summary

Regulation and maximization of lipase production in a mutant derivative of *R. oligosporus* has been investigated using different substrates, inoculum sizes, pH of the medium, temperature, and nitrogen sources in shake flask experiments and batch fermentation in a fermentor. The production of intracellular lipase was improved 3 times following medium optimization involving one-at-a-time approach and aeration in the fermentor. Interestingly, intracellular lipase was poorly induced by oils, instead its production was induced by sugars, mainly starch, lactose, sucrose, xylose, glucose and glycerol. Dependent variables studied were cell mass, lipase activity, lipase yield, lipase specific and volumetric rate of formation. It was confirmed that lipase production in the derepressed mutant is sufficiently uncoupled from catabolite repression. The results of average specific productivities at various temperatures worked out according to the Arrhenius equation revealed that mutation decreased the magnitude of enthalpy and entropy demand in the inactivation equilibrium during product formation, suggesting that mutation made the metabolic network of the organism thermally more stable. The highest magnitudes of volumetric productivity (Q_P=490 IU/(L·h)) and other product attributes of lipase formation occurring on optimized medium in the fermentor are greater than the values reported by other workers. The purified enzyme is monomeric in nature and exhibits stability up to 80 °C and pH=6.0–8.0. Activation energy, enthalpy and entropy of catalysis at 50 °C, and magnitudes of Gibbs free energy for substrate binding, transition state stabilization and melting point indicated that this lipase is highly thermostable.

Key words: lipases, *Rhizopus oligosporus*, oils, casein, ammonium nitrate, ammonium oxalate, starch, glycerol

Introduction

Lipases (EC 3.1.1.3) catalyse the hydrolysis of ester linkages of long chain triglycerides at an oil-water interface. They are ubiquitous enzymes but only microbial lipases are commercially significant due to their wide variety of properties and because of the relatively simple methods of their production in fermentors and recovery from the fermentation broth (*1*). Lipases find use in a variety of biotechnological fields such as food and dairy,

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detergents, cosmetics, pharmaceuticals, agrochemicals (insecticides, pesticides) and oleochemicals (*2*). They have a special contribution in waste management and improvement of tanning techniques (*2,3*). Many bacteria, filamentous fungi and yeasts have the ability to produce lipases (*3,4*). Filamentous fungi have a greater potential for production of extracellular lipases, which are generally used as purified enzymes. Due to the instability of extracellular lipases and costly purification procedures, their application is limited. Direct use of compact cells for intracellular production of lipases or fungal cells immobilized within porous biomass support particles as a whole biocatalyst represents an attractive process for bulk production of biodiesel and polyesters (*5–8*). They are economical because they do not necessarily require purification or further immobilization (*5*).

Moulds produce lipases of high catalytic activity and thermal stability (*4,9*), which are desired characteristics of most of the industrial enzymes. Among strains of *Rhizopus* spp., *R. delemar, R. oryzae, R. arrhizus, R. chinensis* and *R. niveus* have been extensively characterized for production of lipases (*4–8*), but *R. oligosporus* received minor studies (*9*). It is inevitable to investigate other microbial resources as potential producers of lipases of commercial significance. In order to achieve higher enzyme titres, a number of factors need to be optimized, including a suitable organism, process parameters and genetic manipulations (*10–12*).

Biosynthesis of lipase is markedly dependent on the type of inducer used. Lipases have mostly been produced using induction by low-cost substrates, namely, wheat bran, oil cake or wastes from plant oil industries (*4*). In industrial scale fermentation, their use may cause inconsistencies in medium homogeneity, oil-water surface problems and may involve expensive purification process (*4*). Therefore, their constitutive production by an efficient microbial system that can utilize soluble sugars as carbon and energy sources is quite attractive. Suitable mono-, di- or polysaccharides need to be screened to find the best one to support maximum formation of lipases.

Lipases can be produced in both solid-state (SSF) and submerged fermentations. Submerged fermentation is an attractive method since all process variables can be easily controlled, while in SSF the lower mass transfer rate of gas and nutrient diffusion may occur at lower temperature (*4*). The production process for lipases has not been commercialized in Pakistan, but the country imports large amounts of lipases for industrial application. There is currently interest to permit its production locally. The present investigation has been undertaken to investigate the relationship of the substrate, nitrogen source, initial pH of the medium and temperature of fermentation on the regulation of intracellular lipase production to select the best conditions for active lipases (*13*), since medium composition significantly affects the yield and productivity of lipases at industrial scale. For this purpose, a deoxyglucose-resistant (DG^r) mutant derivative (*14*) of a locally isolated strain of *R. oligosporus* was developed and one putative mutant was subsequently evaluated in media containing different substrates and nitrogen sources for indigenous production of lipases.

Materials and Methods

Microorganism

The culture of a native strain of *R. oligosporus* was collected from our culture collection. The culture was maintained on potato dextrose agar (PDA) slants and plates at 4 °C and subcultured every month. The fungus was resubcultured onto PDA plates at ambient temperature for 15 days before use as a source of inoculum. The culture was grown on medium containing (in g/L): peptone 30, glucose 10, K_2HPO_4 2, KCl 0.5, $NaNO_3$ 0.5, $MgSO_4$ 0.5 unless mentioned otherwise.

The spore suspensions ($2 \cdot 10^9$ colony forming units (CFU) per mL) were appropriately diluted and spread on the PDA plates. The agar plates were irradiated with UV light for 5–15 min at a distance of 30 cm by using a germicidal lamp to give a survival ratio of cells of 0.01–0.10 %. The irradiated cells were allowed to grow in the presence of 0.6 % deoxyglucose (DG)+0.5 % tributyrin in the above medium to isolate de-repressed mutants as described earlier (*15*). After 5 days, the colonies showing bigger halo zones were picked up and maintained on PDA-DG slants at 4 °C.

Substrates

Different carbon sources, namely glucose, xylose, glycerol, sucrose, maltose, lactose and starch (10 g/L), were tested for their impact on intracellular lipase production. Starch was optimized for enhanced lipase production. Other process variables like time of fermentation and inoculum size were optimized in the shake flask, while temperature and nitrogen sources were optimized in the fermentor as described earlier (*16*). For this purpose, traditional classical method was used varying one parameter at a time by maintaining preoptimized submerged conditions in shake flasks or fermentor.

Inoculum preparation

A volume of 10 mL of saline was added to 15-day-old culture plates. Spore suspension (containing approx. 10^7 spores/mL) was prepared by scratching the plates. Initially, predetermined volumes of spore suspension were transferred aseptically to each flask containing the fermentation medium (20 mL). A volume of 1 mL of inoculum was found sufficient to support rich growth and enzyme production and was used in other studies keeping other variables constant.

Fermentation

The ability of carbon sources (Table 1) to regulate improved production of lipase in *R. oligosporus* DGM 31 was essentially examined in the above medium. The carbon sources were added individually to batches of basal medium to give a substrate mass level of 10 g/L (found optimum). All media were adjusted to pH=7.0 with 1 M NaOH or 1 M HCl.

Time course (Fig. 1) of lipase production in shake flask batch cultures was carried out at 30 °C in a gyratory shaker (150 rpm). Sample flasks (done in triplicate) were withdrawn after predetermined time intervals (h) and processed. Growth, protein production and intracellular enzyme activities were assayed. Cells were ob-

Table 1. Potential of *R. oligosporus* overproducing mutant derivative for intracellular lipase production from different substrates added to the culture medium (pH=7.0) at 30 °C in shake flasks

Growth substrate	$Y_{P/X}$ IU/g	$Y_{P/S}$ IU/g	Q_P IU/(L·h)	$Y_{X/S}$ g/g
Glucose	1050[c]	420[c]	127.0[c]	0.40[e]
Xylose	1048[c]	440[c]	160.0[c]	0.42[d]
Maltose	1067[b]	510[b]	165.0[b]	0.45[b]
Lactose	726[f]	312[f]	103.6[g]	0.43[cd]
Sucrose	795[e]	350[d]	108.1[f]	0.44[bc]
Starch	1157[a]	590[a]	223.7[a]	0.51[a]
Tween 80	917[d]	330[e]	112.0[e]	0.36[b]
Olive oil	921[d]	350[d]	115.1[d]	0.38[f]
F-value	293.90	304.40	289.37	156.39
p-value	0.000	0.000	0.000	0.000

$Y_{P/X}$, $Y_{P/S}$, Q_P and $Y_{X/S}$ are specific enzyme yield, enzyme yield per g of substrate utilized, volumetric rate of enzyme production and cell yield, respectively. Each value is a mean of three replicates. Values followed by different superscript in columns for $Y_{P/X}$, $Y_{P/S}$, Q_P and $Y_{X/S}$ differ significantly at $p \le 0.05$ by 6.376, 0.354, 0.414 and 0.017, respectively, using Duncan's multiple range test applying ANOVA 2 in MSTAT-C software and degree of freedom of 23 and error mean square of 14.245, 0.044, 0.060 and 0.0001 for $Y_{P/X}$, $Y_{P/S}$, Q_P and $Y_{X/S}$ columns, respectively

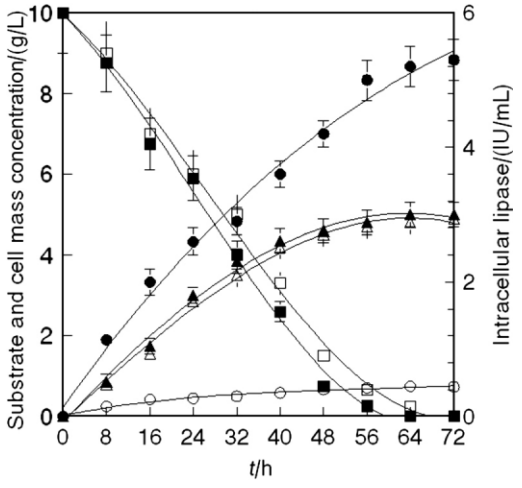

Fig. 1. Representative time course of intracellular lipase production (○,●) and cell mass formation (△,▲) following the growth of the wild organism (○,□,△) on glucose (10 g/L, □,■) and mutant derivative (●,▲,■) in the basal medium (pH=7.0) at 30 °C in shake flask cultures (150 rpm). Each point is a mean of three independent experiments. Bars indicate standard deviation among three replicates. Though cell mass and substrate consumption rates were comparable, lipase produced by mutant derivative differed significantly at $p \le 0.05$

tained by centrifugation (15 000×*g*, 15 min at 4 °C) of the above fermentation broth. The cell-free supernatant was preserved for protein assays and cell pellets were washed twice with saline, suspended in 10 mL of phosphate buffer (pH=7) containing glass beads (0.45 μm diameter) and shaken vigorously at 200 rpm for 15 min, or alternatively, the cells were ultrasonicated as described earlier (*17*). The contents were centrifuged and the supernatant (cell extract) was assayed for the intracellular lipase activity. Extracellular and intracellular lipase activities (in IU/mL) were almost the same, and the extracellular enzyme activities were not presented. Dry cell mass was determined as described previously (*15*).

Effect of pH on lipase production

The effect of initial pH of the medium was observed in the range of 5.0–10.0 (Fig. 2). The pH was adjusted with 1 M NaOH or 1 M HCl, keeping other variables constant. The optimum pH of the medium achieved by this step was kept constant for all subsequent experiments.

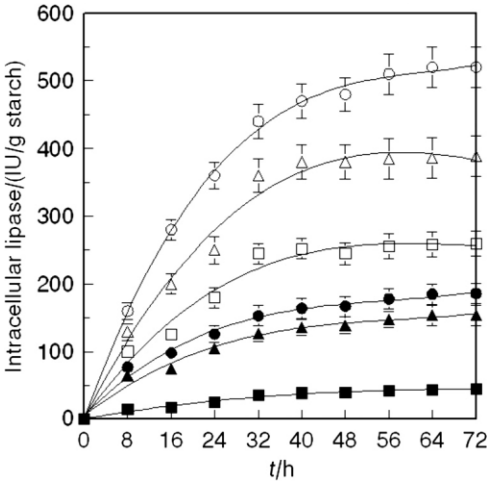

Fig. 2. Effect of the initial pH of the basal medium on intracellular lipase production by the mutant derivative. Initial pH of the medium varied from pH=4.0 to 9.0 (▲ pH=4, ● pH=5, △ pH=6, ○ pH=7, □ pH=8, ■ pH=9). Fermentation temperature was maintained at 30 °C in a rotary shaker (150 rpm), inoculum size was 10 %. Each point on the graph is a mean of three experiments. Error bars show standard deviation among replicates. All values differed significantly at $p \le 0.05$

Effect of inoculum size on lipase production

The inoculum ($2.5 \cdot 10^7$ spores/mL) was varied from 0.5 to 3.0 mL to see the effect on the lipase production when all other variables were kept constant.

Effect of temperature on lipase production

The effect of incubation temperature was observed in the range of 15–45 °C in a shaking incubator, keeping other variables constant. The results obtained at different temperatures were used to estimate the thermodynamic parameters both for lipase production and thermal inactivation of the metabolic network during enzyme synthesis. For this purpose, empirical Arrhenius equation (*18*) was used to describe the relationship between temperature dependent reversible and irreversible inactivation of lipase production for temperature range of 15–45 °C. Specific rate (q_p, IU/(g dry cells·h)) of product formation (Eq. 1) was used to calculate these attributes:

$$q_P = k_B T/h \cdot e^{\Delta S^*/R} \cdot e^{\Delta H^*/RT} \qquad /1/$$

$$\ln(q_p/T)=\ln(k_B/h)+\Delta S^*/R-\Delta H^*/RT$$

where plot of $\ln(q_p/T)$ against $1/T$ gave a straight line whose slope was $-\Delta H^*/R$, and the intercept was $\Delta S^*/R+\ln(K_B/h)$, and Planck constant $h=6.63\cdot10^{-34}$ Js and Boltzman constant $K_B=1.380658\cdot10^{-23}$ J/K.

Rate of lipase production in 14-litre fermentor

Erlenmeyer flasks were inoculated with 5 % (by volume) spore suspension (10^7 spores/mL) from a 3-day-old PDA slant and cultivated at 30 °C and 150 rpm for 24 h on an orbital shaker. The precultures were used as inoculum (10 %, by volume) for the 14-litre stirred fermentor (CelliGen 310, New Brunswick, Edison, NJ, USA) with a working volume of 10 L, fitted with automatic pH, temperature, dissolved oxygen tension (DOT), agitation and airflow rate controls. The optimized medium (10 L) was steam-sterilized *in situ* for 30 min. The medium was inoculated with seed culture (10 % inoculum, by volume) prepared as above. Silicone oil was used as an antifoaming agent. The pH of the culture was controlled at pH=7.0. The fermentor temperature was maintained at 30 °C. The corresponding aeration rate was adjusted to 0.5, 0.75 and 1 vvm. Dissolved oxygen was measured polarographically. Foaming was controlled by automatically adding antifoam when required. Samples in triplicate were collected periodically to follow the assay of dry cell mass, protein, and enzyme activity.

Purification of enzyme

Intracellular lipase was purified by a combination of ammonium sulphate precipitation, ion exchange and gel filtration techniques (*4,10*). All the purification steps were performed at 4 °C unless otherwise indicated. The crude intracellular extract was concentrated by dissolving in one tenth of the original volume. The concentrated enzyme sample was fractionated on DEAE (diethylaminoethyl)-cellulose column (1.6×10 cm) equilibrated with Tris-HCl buffer (pH=8.5) at a flow rate of 1.0 mL/min. The bound proteins were eluted by a linear gradient of 0–0.5 M NaCl in Tris-HCl buffer (pH=8.5). Fractions (2 mL) were collected using a fraction collector FRAC-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) and assayed for protein and lipase activity. Active fractions containing enzyme activity were pooled, dialyzed and freeze-dried for further experiments.

The enzyme sample from the above step was dissolved in one tenth of the original volume of distilled water and applied to Sephadex G-100 column (1.6×60 cm) pre-equilibrated with 0.05 M phosphate buffer (pH=7.0). Elution was done with the same buffer at a flow rate of 1.5 mL/min. Fractions (2.5 mL) were analyzed for protein and lipase activity. Active fractions were pooled and assayed for protein content and lipase activity.

Lipase assays

Lipase activity in the cell extract was determined titrimetrically according to Gilbert *et al.* (*16*). The substrate contained 5 mL of olive oil emulsion (10 mL of olive oil+2 g of gum arabic+90 mL of hot distilled water), diluted to 10 % (by volume) in 0.14 M Na_2CO_3 and 0.05 M $CaCl_2$. The reaction was started by adding 1 mL of appropriately diluted crude enzyme extract to the substrate mixture at pH=6.0 and incubated for 15 min at predetermined temperatures. Liberated fatty acids were titrated against freshly prepared 0.05 M NaOH. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of fatty acid from olive oil per min under the specified assay conditions.

Alternatively, lipase activity was assayed spectrophotometrically using *p*-nitrophenyl palmitate (*p*NPP) as substrate. Enzyme solution (50 μL) was added to 950 μL of the substrate solution consisting of one part solution A (5.0 mM *p*NPP in 2-propanol) and nine parts solution B (100 mM potassium phosphate buffer (pH=7), 0.4 % Triton X-100 and 0.1 % gum arabic), which was freshly prepared before use. The reaction mixture was incubated at 37 °C for 20 min and stopped by boiling for 10 min, followed by centrifugation at 8000×*g* for 10 min. The release of *p*-nitrophenol was measured at 410 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per min. Total number of enzyme units per mL of enzyme preparation measured by both methods was equal; therefore, titrimetic method was preferred because of its simplicity and repeatability showing linear relationship between $1/V$ and $1/c$(olive oil). Lipase activity was also determined by using *p*-nitrophenyl butyrate and tributyrin, but lipase activity in units per mL of intracellular and extracellular enzyme preparations was significantly different (25 % of that on *p*NPP or olive oil), so its use was ignored.

Determination of kinetic parameters

Kinetic data for enzyme production were collected as described earlier for *Aspergillus niger* (*19*).

Results and Discussion

Substrate regulation of intracellular lipase production

Carbon is the main component of cells and carbon sources play an integral role in the synthesis and regulation of enzyme production. There is an increasing recognition that the elemental composition of enzymes can be related to carbon source to support the formation of nucleic acids, amino acids and the proteins they code. There exists a strong and positive correlation between N/C values of genomes and proteomes of different organisms. Therefore, there is a compelling need to initially screen different carbon sources (Table 1) for their ability to regulate lipase production on DG^r mutant derivative *R. oligosporus* DGM 31 using other optimal operating variables from literature (inoculum size 10 %, temperature 30 °C, initial pH=7, and basal medium described earlier). All carbon sources supported markedly different titres of intracellular lipase activities measured on olive oil. The number of units per mL of enzyme preparation for lipase activity was equal on olive oil (Table 1) and *p*-NPP (data not shown). The influence of treatments on all fermentation attributes of lipase production was highly significant as revealed by *F*- and p-values at $p\leq0.05$ (Table 1).

The mutant DGM 31 surprisingly showed maximum enzyme production stimulated by the presence of the non-lipid compounds (starch), followed by lactose, xylose and glucose. Synthesis of lipase in the presence of glucose confirmed that lipase production in DG^r mutant was not related to catabolite repression. Incorporation of lipids has been shown to increase lipase yield in many cases (*4*), but it must be emphasized that the results reported in literature in this respect are not equivocal. In contrast to the aforesaid investigation, mutant DGM 31 did not show good lipase activity in the media containing lipids, which is in accordance with studies reported by Nahas (*9*). Maximum cell mass yield was regulated by starch, followed by maltose. This suggested that increased production in the mutant was correlated with cell mass formation. Enhanced cell mass formation by *R. oligosporus* DGM 31 could lead to increased productivity of lipases in large-scale fermentors.

Time course studies of lipase production by both wild and mutant organisms from glucose (representative substrate) revealed that after 48 h the wild organism supported only the basal level of activity, while the mutant organism supported maximum lipase synthesis. Application of Luedeking and Piret model using specific rate of lipase formation indicated that product formation was both growth and non-growth associated and lipase was not a purely primary metabolite. However, a good relationship existed between the enzyme titres and cell mass formation. Maximum volumetric rate of lipase formation (Q_P) occurred on starch (223.7 IU/(L·h)) followed by maltose and glucose. D'Annibale *et al*. (*20*) obtained maximum volumetric lipase productivity of 5.4 IU/(L·h) using *Penicillium citrinum* in unoptimized medium.

Effect of pH on lipase production

The initial pH of the medium regulates product formation. Initial pH conditions were optimized for maximum production of lipase. Maximum activity by *R. oligosporus* over-producing mutant (Fig. 2) was observed at pH=7.0 in 48 h at 30 °C with inoculum size of 10 %. Some basic compounds accumulated in the fermentation medium and led to the increase in medium pH (to pH= 8.0), but when initial pH was above 8.0, there was delayed production of lipase. Alkaline extracellular pH could enhance intracellular pH of the cells and destabilize the enzyme synthetic network since equilibrium in enzyme catalyzed reactions is reached at specified intracellular pH values (*21*) and intracellular ionic strength of the biological systems. For other lipase-producing organisms, namely *Candida rugosa* (*1*), *A. niger* and other fungi (*4*,*11*,*13*,*20*), different media of similar or higher pH supported maximum production of lipases.

Effect of inoculum size

Data in Fig. 3 show the regulation of enzyme synthesis by inoculum size in time course study. Maximum production ((1156±12.1) IU/g dry cells) occurred with 5 % inoculum size. Lower or higher inoculum size did not support higher activity. Inoculum density is an important variable in submerged processes since higher inoculum levels increase spore concentration, thereby inhibiting fungal growth and enzyme induction (*20*). With the increase in mycelium mass, the production of enzyme declined due to enhanced cell mass formation, resulting in the exhaustion of nutrients from the fermentation mash. Lower inoculum may enhance time of fermentation to get maximum enzyme yields. Thus 5 % inoculum size regulated higher enzyme synthesis and was used in subsequent experimental work. In contrast to our findings, 10 % inoculum level for maximum lipase production on olive mill wastewaters had been reported previously (*20*).

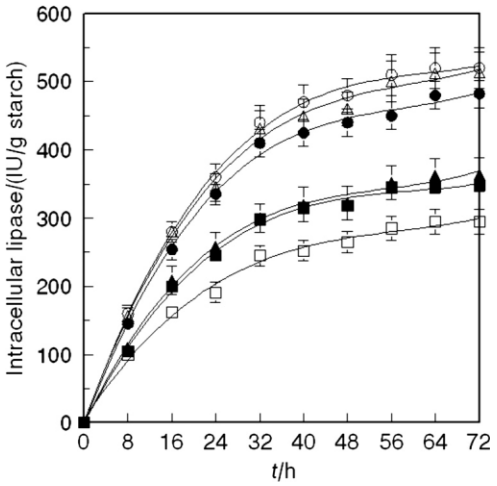

Fig. 3. Effect of inoculum size on intracellular lipase production. Initial pH of the medium was 7.0 and temperature was maintained at 30 °C in a rotary shaker (150 rpm). Inoculum size varied from 2.5 to 15 % (○ 2.5 %, △ 5.0 %, ● 7.5 %, ■ 10 %, ▲ 12.5 %, and □ 15 %). Each point on the graph is a mean of three experiments. Error bars show standard deviation among replicates. All values differed significantly at $p \leq 0.05$

Effect of temperature

The most desirable property of new developed industrial strains is their genetic stability and physiological reproducibility (*14*). To assess the mutational effect on thermostability of the genetic make up of the DG^r mutant organism, the inoculated fermentation medium was incubated at 15, 20, 25, 30, 35, 40 and 45 °C (Fig. 4) in shake flasks (see Materials and Methods), keeping all other fermentation conditions constant. Normally, high temperature can cause inactivation of enzymes of the metabolic pathway, while low temperature may not permit flow of nutrients across cell membrane, resulting in high demand for maintenance energy (*18*) for lipase production by the organism. There was a progressive increase in the average specific productivity of intracellular lipase when the temperature was increased from 15–30 °C. Maximum enzyme specific productivity (357 IU/(g dry cells·h)) was supported at 30 °C. This temperature is in the normal range reported for other moulds (*4*,*7*,*11*). Conversely, specific productivity progressively decreased over 30 °C, although maximal growth occurred at 35 °C. Reduced enzyme productivity was probably due to the well-known thermal inactivation of metabolic network at temperatures higher than the optimum.

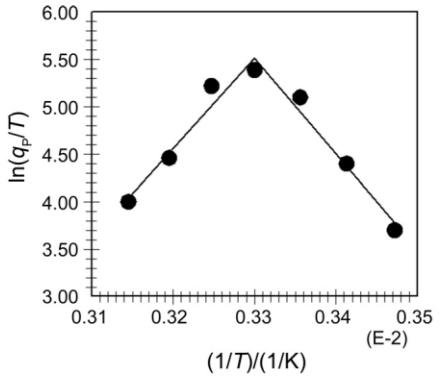

Fig. 4. Arrhenius relationship (between specific productivity q_p/T and $1/T$, where T is absolute temperature) used to calculate the values of both enthalpy and entropy of intracellular lipase formation and its inactivation during growth of *R. oligosporus* mutant derivative DGM 31 on starch medium (initial pH=7.0, inoculum 5 %) at different temperatures in shake flask culture studies. Each point is a mean of three experiments. The value of standard deviation among replicates was too small to be visible. All values differed significantly at $p \leq 0.05$

Under controlled environmental conditions, thermodynamic parameters are the driving force for microbial growth and product formation (*21*). Low magnitudes of enthalpy, entropy and Gibbs free energy indicate the stability of the metabolic network. Arrhenius model was used to calculate enthalpy and entropy of product formation during growth of the organism to demonstrate the hidden thermal process regulatory mechanism (*21*, *22*). The optimum specific productivity was addressed from the interception of two straight lines (Fig. 4), representing increasing and decreasing specific productivity magnitudes around the optimum. Slopes were used to calculate the values of enthalpy of activation/inactivation equilibrium, while the intercept values were used to calculate the entropy demands of activation/inactivation equilibrium. The results indicate that the activation enthalpy of lipase formation by mutant cells (80.5 kJ/mol) was lower than ethanol and α-galactosidase production by thermotolerant yeasts, as reported earlier (*22*,*23*). The activation entropy of lipase formation equilibrium by the mutant (58.3 J/(K·mol)) was lower than that by the thermostable organisms (*23*,*24*). This suggests that the genetic make up of the mutant derivative was thermodynamically more stable comparable to other thermostable organisms.

When organism was grown at 37–40 °C, thermal inactivation of the metabolic network was an expected phenomenon. The thermal inactivation of enzymes of the metabolic network is accompanied by the disruption of noncovalent linkages, including hydrophobic interactions of enzymes of the metabolic network, with concomitant increase in the enthalpy of activation (*25*). The opening up of the enzyme structures of metabolic network is accompanied by an increase in the disorder, or increase in entropy of activation (*26*). The value of thermodynamic parameter of inactivation calculated from Fig. 4 gave a magnitude of activation enthalpy (ΔH_D^*) of thermal inactivation for the mutated cells of 74 kJ/mol and was lower than that for its production (80.5 kJ/mol). This means that its rate of deactivation did not increase faster with the increase in temperature, indicating its conformational stability. It was also significantly lower than the values obtained with other microbial systems (160–235 kJ/mol) (*23*). The activation entropy value of thermal inactivation by the mutant culture was also very low (–444.4 J/(K·mol)), it had negative symbol and was found to be comparable with that of α-amylase from *Bacillus licheniformis*, as revealed by its negative ΔS^* (–150 J/(K·mol)) at 80 °C (*26*). This suggests that this inactivation phenomenon implied no deactivation in the enzyme production pathway during growth of DG^r mutant on the substrate up to 45 °C. This also indicates that the mutant organism acquired the ability to exert a sort of protection against thermal inactivation, most probably by acquiring chaperones, which assisted the folding of protein within the cells (*27*), thus supporting our assumption that the mutation stabilized the biological system of mutant cells during production of lipases. It is in contrast to previous findings that mutation made the enzyme thermolabile (*14*), but it supports the assumption that DG-resistance modulates the transport mechanism of enzymes and causes posttranslational modifications (*14*) by putting polysaccharides on protein ensemble and could have induced thermostability during product accumulation.

Effect of nitrogen sources

Nitrogen sources, including inorganic and organic nitrogen sources, play a pivotal role in regulating synthesis of hydrolases. Inorganic nitrogen sources are consumed quickly and normally cause repression of enzyme synthesis due to formation of ammonium repressible entity (AreA protein) (*28*), while organic nitrogen sources can supply amino acids, and many cell growth factors, which are needed for cell metabolism and protein synthesis. Therefore, both inorganic and organic nitrogen sources were used to observe their regulatory role in lipase fermentation in shake flask cultures. The dependence of lipase production on nitrogen sources is presented in Table 2. The influence of all nitrogen sources on all fermentation attributes (kinetic parameters) of lipase production was highly significant (Table 2) as revealed by *F*- and p-values at $p \leq 0.05$. Ammonium oxalate, ammonium nitrate and casein were optimal nitrogen sources for upregulation of intracellular lipase synthesis (up to 2-fold over the basal medium). The activities on media containing urea, peptone or corn steep liquor were significantly lower ($p \leq 0.05$) than those with ammonium nitrate. Ammonium sulphate was another nitrogen source having stimulatory influence on lipase regulation after casein.

Rate of synthesis in shake flasks and well-aerated fermentor

Scaling up of the experiment is crucial for any study related to fermentation that is aimed at the hyperproduction of a particular enzyme. Production of lipases by *R. oligosporus* is an aerobic process requiring sufficient mass of dissolved oxygen in the medium. It has been previously reported (*29*) that when dissolved oxygen concentration falls below a critical level, an instant decrease in lipase biosynthesis occurs. Under the aeration

Table 2. Dependence of lipase production by *R. oligosporus* on nitrogen sources in culture medium (pH=7.0) at 30 °C in shake flask culture studies

Nitrogen source	$Y_{P/X}$ IU/g	$Q_{P/S}$ IU/(g·h)	Q_P IU/(L·h)	q_P IU/(g·h)
NH_4Cl	1242[e]	20[g]	198[g]	328[c]
NH_4NO_3	1680[b]	33[b]	327[b]	312[d]
$(NH_4)_2SO_4$	1467[d]	25[f]	220[f]	285[e]
NH_4-oxalate	1786[a]	35[a]	348[a]	336[b]
Peptone	1156[g]	27[d]	256[e]	231[h]
Yeast extract	1464[d]	26[e]	276[d]	234[g]
Casein	1564[c]	30[c]	299[c]	355[a]
Corn steep liquor	1225[f]	19[h]	195[h]	251[f]
Urea	1090[h]	15[i]	185[i]	225[i]
F-value	321.21	216.14	312.60	314.61
p-value	0.000	0.000	0.000	0.000

$Y_{P/X}$, $Q_{P/S}$, Q_P and q_P are specific enzyme yield, volumetric enzyme rate based per g of substrate utilized per h, volumetric rate of enzyme production per L per h and specific enzyme productivity, respectively. Each value is a mean of three replicates. Values followed by different superscript in columns $Y_{P/X}$, $Q_{P/S}$, Q_P and q_P differ significantly at $p \leq 0.05$ by 3.275, 0.509, 0.401 and 0.352, respectively, using Duncan's multiple range test applying ANOVA 2 in MSTAT-C software with degree of freedom of 26, and error mean square of 3.807, 0.092, 0.057 and 0.044 for the above columns, respectively

rate of 0.5 vvm (optimized), fermentation was performed with different agitation speeds of 200, 300 and 400 rpm in the 14-litre fermentor cultures. Activities of lipases obtained from the mutant cultures at an agitation speed of 300 rpm were the highest (Fig. 5). At lower agitation rate (200 rpm), oxygen limitation occurred (results not presented), thereby suppressing fungal growth and enzyme synthesis, but DOT was maintained at 20 % saturation throughout the fermentation with agitation speed of 300–400 rpm (and was found optimum). Lipase production was associated with fungal growth and reached maximum activity in 48 h.

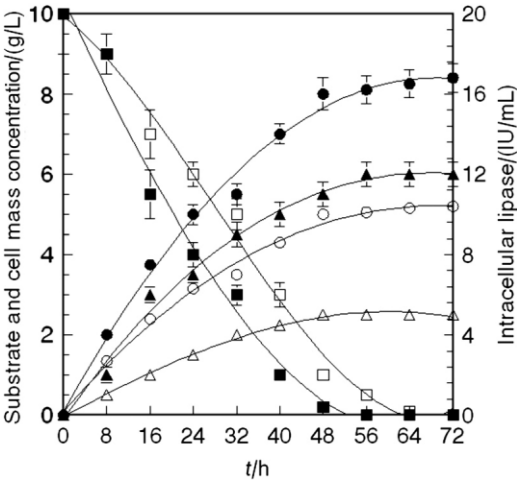

Fig. 5. Time course of lipase production (○,●) and cell mass formation (△,▲) following the growth of mutant derivative on starch (10 g/L, □,■) supplemented with ammonium oxalate in optimized medium (pH=7.0) at 30 °C in shake flask cultures (150 rpm) (○,△,□) and fermentor (●,▲,■). The medium was inoculated with 5 % inoculum size. Each point is a mean of three independent experiments. Bars indicate standard deviation among the replicates. All values differed significantly at $p \leq 0.05$

Potential kinetic parameters for production of lipase in *R. oligosporus* during growth in optimized media in shake flask and fermentor studies (Table 3) indicated that starch supplemented with ammonium oxalate supported 3-fold higher specific productivity than that by unoptimized medium (Table 1), and that fermentor aeration enhanced enzyme titre by 1.55-fold over optimized media in shake flasks. Furthermore, the cell mass productivity (0.2 g/(L·h)) was 1.33-fold and substrate consumption rate (0.288 g/(L·h)) was 2.76-fold higher than those in the shake flask. The influence of treatments on

Table 3. Dependence of intracellular lipase production on culture conditions, namely shake flask and fermentor cultures under optimized conditions: kinetic parameters for substrate consumption and lipase formation by *R. oligosporus* mutant derivative DGM 31

Parameters	Shake flask	Fermentor	Mean	*F*-value	p-value
Substrate consumption parameters					
μ_m/h^{-1}	0.11[c]	0.15[a]	0.13[b]	1681	0.000
Q_S/(g/(L·h))	0.104[c]	0.288[a]	0.20[b]	761	0.000
Q_X/(g/(L·h))	0.15[c]	0.20[a]	0.18[b]	70.0	0.000
$Y_{X/S}$/(g/g)	0.50[c]	0.60[a]	0.55[b]	225.0	0.0001
q_S/(g/(g·h))	0.22[c]	0.25[a]	0.24[b]	230.5	0.0000
Product formation parameters					
Q_P/(IU/(L·h))	314[c]	490[a]	402[b]	690.64	0.000
$Y_{P/S}$/(IU/g)	1040[c]	1650[a]	1345[b]	71.57	0.000
$Y_{P/X}$/(IU/g)	2080[c]	2750[a]	2415[b]	305.37	0.000
q_P/(IU/(g·h))	229[c]	413[a]	321[b]	265.52	0.0000

Q_X, Q_S, $Y_{X/S}$ and q_S are volumetric rate of dry cell mass formation, volumetric rate of substrate consumption, dry cell mass yield, and specific rate of substrate consumption, respectively. For other symbols, see Tables 1 and 2. Each value is a mean of three replicates. Values followed by different superscripts in rows differ significantly at $p \leq 0.05$ using Duncan's multiple range test applying ANOVA 2 in MSTAT-C software

all fermentation attributes of lipase production was highly significant (Table 3) as revealed by *F*- and p-values at $p \leq 0.05$. The values of the kinetic parameters obtained for lipase are several-fold higher than the values reported by other workers on *R. oligosporus*, *R. oryzae*, *Aspergillus niger* and their mutants (*11*,*30*), *Penicillium citrinum*, *Geotrichum candidum*, *R. arrhizus* and *Fusarium solani* (*4*,*9*,*13*, *31*,*32*), and yeasts (*1*,*3*,*4*,*12*,*31*,*33*). The enzyme volumetric productivity after growth on starch with ammonium nitrate as the nitrogen source was 34.83 IU/(g substrate·h). This value is higher than the values reported by other workers: 1.95 IU/(g substrate·h) (*33*), 0.91 IU/(g substrate·h) (*4*) and 5.05 IU/(g substrate·h) (*34*). Specific productivity of lipases (Table 3) was comparable to the lipase production by recombinant strain of *Aspergillus niger* expressing a lipase-encoding gene from *Thermomyces lanuginosus* (*35*), while enzyme regulatory process was comparable to that exhibited by a *Bacillus* sp. (*36*). Enhancement in lipase production was a consequence of alteration in genes related to all activities, namely lipase, hexokinase or DOG-6P phosphatase as reported in DG^r mutants (*37*).

Purification and biochemical characterization of lipase

Three-step purification technique (see Materials and Methods) made the enzyme free from all other proteins and the final percentage recovery was 49.8 %. The specific activity of pooled fractions increased from 30 to 65 IU per mg of protein after gel filtration chromatography. The purified enzyme was homogenous on SDS-polyacrylamide gel (Fig. 6) with a subunit molecular mass of

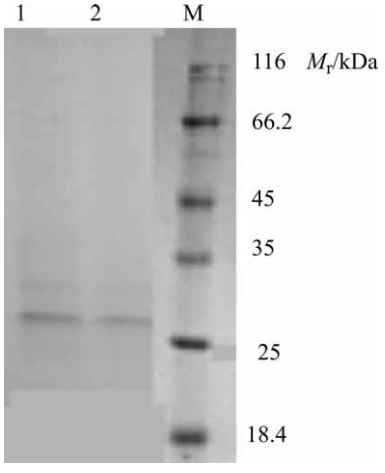

Fig. 6. Polyacrylamide gel electrophoresis of purified lipases of wild and mutant strains of *R. oligosporus*. Electrophoresis was performed on 12 % polyacrylamide gel in the presence of 0.1 % SDS. Lane 1, purified lipase derived from the parental strain; lane 2, purified lipase derived from the mutant; lane M, molecular mass marker proteins

29.5 and 31.6 kDa for mutant- and parent-derived enzymes, respectively. This was attributed to a small deletion in the gene for the enzyme acquired by mutant organism. The enzymes also exhibited a molecular mass of 28 and 29.5 kDa, respectively on Superose gel filtration column, suggesting that the enzyme was monomeric in nature. *R. oligosporus* lipase is similar to the lipase from *R. oryzae* (*10*), *Penicillium candidum* (*38*), *R. chinensis* (*39*) and *P. expansum* (*40*). The above molecular masses are lower than those of lipases from *P. cyclopium* (*41*) and *Geotrichum marinum* (*42*). Optimal pH of purified enzyme was 8.0 for the mutant organism (Table 4) and it was the same as for that from *G. marinum* (*42*), but higher than that for *P. cyclopium* (pH=7.0) (*41*) and its wild culture (Table 4). Protein stability was measured in buffers with different pH values as a function of time (Fig. 7a). Purified enzyme from *R. oligosporus* was stable over the pH range of 6.0–9.0 (80–100 %). Lipases that are more active in the pH range of 6.0–10.0 can act as promising agents in detergent industries (*4*).

Table 4. Biochemical properties of purified lipases derived from wild and mutant derivative of *R. oligosporus* at 50 °C

Parameters	Values of lipases derived from organisms	
	Mutant	Wild type
v_{max}/(μmol/(mg·min))	166	132
K_{cat}/(min^{-1})[a]	4486	3626
K_m/mM	1.9	2.8
K_{cat}/K_m	2361	1295
Native M_r/kDa	29.5	31.6
Subunit M_r/kDa	28.0	29.5
E_a/(kJ/M)	32.5	48.2
pH optimum	8.0	7.0
ΔG^*/(kJ/mol)	55.2	56.7
ΔH^*/(kJ/mol)	29.8	45.5
ΔS^*/(J/mol)	–78.63	–34.67
$\Delta G^*_{E\text{-}T}$/(kJ/mol)[b]	–20.85	–19.24
$\Delta G^*_{E\text{-}S}$/(kJ/mol)[c]	1.72	2.76

Each value is a mean of three observations. Standard deviation among replicates was between 5 and 7.5 % and has not been shown

[a] $K_{cat}=v_{max}$/mM of enzyme

[b] $\Delta G^*_{E\text{-}T}$ (free energy of transition state binding)$=-RT\cdot\ln(K_{cat}/K_m)$

[c] $\Delta G_{E\text{-}S}$ (free energy of substrate binding)$=-RT\cdot\ln K_a$, where $K_a=1/K_m$

ΔH^* (kJ/mol)$=E_a-RT$, where E_a is the activation energy for substrate hydrolysis

ΔG^* (kJ/mol)$=-RT\cdot\ln((K_{cat}\cdot h)/(k_B\cdot T))$ and $\Delta S^*=(\Delta H^*-\Delta G^*)/T$

ΔH^*, ΔG^* and ΔS^* are enthalpy, Gibbs energy and entropy of enzyme catalysis, respectively

Purified enzymes from mutant and parental strains exhibited K_m values of 1.9 and 2.8 mM, respectively, while v_{max} values were 166 and 132 μmol/(mg·min). Lower K_m value against *p*NPP determined the higher affinity of the enzyme for substrate. This affinity was better than that (11.4 mM) of lipase from *G. marinum* (*42*) and the wild organism (2.8 mM) (Table 4). Higher value of catalytic efficiency (K_{cat}/K_m) of 2361 (Table 4) suggested that purified enzyme hydrolyzes the substrate very efficiently. However, turnover number (K_{cat}) and catalytic efficiency (K_{cat}/K_m) values of intracellular lipase from other fungi

could not be worked out, but v_{max}/K_m value (87.4) was equal to that (87.0) for *G. marinum* (*42*).

The effect of temperature on lipase activity was determined by assaying activity at various temperatures ranging from 10 to 80 °C as a function of time (Fig. 7b). Maximum catalytic efficiency of lipase was obtained at 50 °C, compared to temperature optimum of 40 °C for lipase from *G. marinum* (*42*). Decline in enzyme activity was found at temperatures above 60 °C and below 20 °C.

The protein mid-point inactivation temperature (T_m), activation energy, conformational stability at elevated temperatures, activation parameters for catalytic activity, transition state binding energy and stability of the native state ensemble are potential determinants for thermostable biocatalysts. To test the thermal stability of the purified lipase, the enzyme was incubated in 0.05 M Tris-HCl buffer (pH=8.0) for the mutant and Tris-HCl buffer (pH=10) for the parent strain with the substrate at various temperatures for 2 h, and the residual activity was determined at 60 °C (Fig. 7b). Purified enzyme retained 90 % of activity when incubated at 60 °C for 120 min. The enzyme was stable up to 70 °C, at which temperature it lost about 45 % of activity in 2 h. In the absence of the substrate, the half-life of purified enzyme at 60 and 70 °C was 78 and 36 min, respectively, and it decreased to 25 min at 80 °C, as shown in Fig. 7c. Thermostability is an important property of enzymes with special reference to lipases, which have a great potential in industrial applications (*2–10*).

Activation energy for catalysis of *p*NPP was 32.5 kJ/mol (Fig. 7d), which is significantly lower than required by other fungal lipases and enzymes from mutated organisms (*26,43,44*). After the inflexion in temperature (Fig. 7d), the enzyme was marginally unfolded. This inferred the thermostability of the test enzyme as reported earlier (*43,44*). The T_m was 80 and 70 °C (Fig. 7b) for the mutant- and wild parent-derived lipases, respectively, which had not been reported previously.

The *R. oligosporus* mutant-derived enzyme required less free energy ($\Delta G^*_{E\text{-}S}$) for substrate binding (1.72 kJ/mol). Similarly, the enzyme released the higher amount (Table 4) of transition state binding energy ($\Delta G^*_{E\text{-}T}$) (–20.85 kJ/mol), as compared to those of other thermostable enzymes (*26,43,44*), signifying that the high catalytic efficiency of this lipase is due to the transition state stabili-

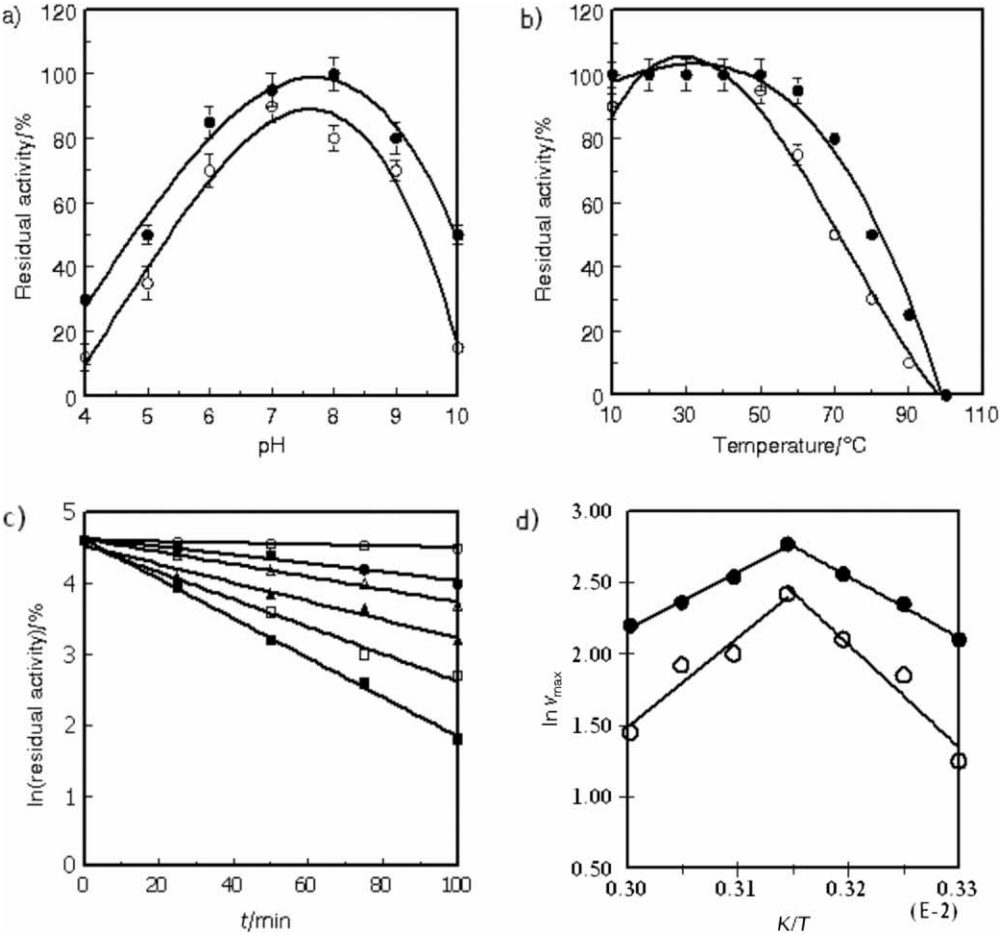

Fig. 7. a) Effect of pH on stability of parent- (○) and mutant-derived (●) intracellular lipases was studied by measuring residual activity after two-hour incubation of the enzyme at pH=4.0 to 10.0 and the activity was expressed in percentage and determined at pH=7.0. b) Temperature stability of the enzyme was studied by measuring residual activity after 2-hour incubation of the enzyme at temperature ranging from 10–95 °C and residual activity was measured at 50 °C. c) Time-dependent inactivation of enzyme was measured at 50 (○), 55 (●), 60 (△), 65 (▲), 70 (□) and 80 °C (■) to determine the half-life of enzymes. d) Represents the determination of activation energy of both enzymes as described previously (*10*)

zation. The activation energy (E_a) profile of the mutated enzyme shows that this lipase has lower E_a demand for *p*NPP hydrolysis up to 80 °C.

This study provided insight into the inherent properties of thermostability of protein ensemble. The enzyme had lower demand of both entropy and enthalpy of *p*NPP hydrolysis (Table 4) as observed for thermostabilized and thermostable enzymes (*26*,*43*,*44*).

Conclusion

A DG^r mutant derivative (*R. oligosporus* DGM 31), developed through UV mutagenesis, was promising and supported high titres of intracellular lipase without supplements. Addition of ammonium oxalate, ammonium nitrate and casein as nitrogen supplements further enhanced enzyme formation rate. Inoculum 5 %, initial pH=7.0, and fermentation temperature of 30 °C for 48 h were optimized in shake flasks. After optimization of the culture medium, the lipase activity increased from 5 IU/mL in the basal medium to 10 IU/mL in shake flasks. Aeration rate in the fermentor enhanced product formation, cell mass formation and substrate consumption rates. Additional additives like yeast extract, ammonium molybdate, casein and ammonium oxalate may further enhance enzyme production in the fermentor and optimized media may be used for industrial production of lipases. Biochemical properties of pure enzyme indicated that it possessed high substrate specificity, lower demand of activation energy, Gibbs free energy for enzyme substrate complex formation and transition state stabilization, as reported earlier *(26*,*43*,*44*).

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