ACTA ABP BIOCHIMICA POLONICA

Vol. 60, No 3/2013
387–393
on-line at: www.actabp.pl

Regular paper

An organic solvent and surfactant stable α-amylase from soybean seeds

Nivedita Jaiswal✉ and Om Prakash

Department of Biochemistry, Faculty of Science, Banaras Hindu University, Varanasi U.P., India

An organic solvent and surfactant stable α-amylase was obtained from soybean seeds. The direct and indirect effect of various organic solvents (non-polar, polar protic, and polar aprotic) and surfactants on the activity and stability of free enzyme was determined. The enzyme showed a very high catalytic efficiency and stabilization against most of the organic solvents and surfactants tested, except for few. Those organic solvents and surfactants (like chloroform, dimethyl formamide, n-butanol, and Tween 20), which caused an inhibition in enzyme activity, were used to study their effects on immobilized enzyme. The inhibitory effect was found to be decreased in immobilized enzyme as compared to free enzyme indicating that immobilization imparted stability to the enzyme. Moreover, the possibility of reuse of the enzyme in the presence of the organic solvents and surfactants was increased upon immobilization. The stability of soybean α-amylase towards organic solvents and surfactants shows that it is a potential candidate for use in organic-solvent biocatalysis as well as in detergent industries.

Key words: agarose, agar, gelatin, biocatalysis, detergent, immobilization

Received: 25 September, 2012; revised: 13 September, 2013; accepted: 19 September, 2013; available on-line: 20 September, 2013

INTRODUCTION

Over the past few decades, the use of enzymes to carry out reaction in organic media has gained considerable interest and has emerged as a major area of biotechnology research and development (Koskinen & Klibanov, 1996). However, native enzymes almost exhibit low activity in organic solvents, often four or five orders of magnitude lower than in aqueous solutions (Serdakowsli & Dordick, 2008). Thus, the enzymes that naturally remain stable in the presence of organic solvents without the need for special stabilization could be very useful for biotechnological applications in which such solvents are used (Ogino *et al*., 2000). Therefore, the search for organic solvent-tolerant enzymes has been an extensive area of research (Sellek & Chaudhuri, 1999). Lipase appears to be the only enzyme, which has received maximum attention in the field of non-aqueous enzymology (Zaks & Klibanov, 1984; Klibanov, 1989; Carrea *et al*., 2000). Organic solvent-tolerant enzymes isolated from various microorganisms have been reported (Ruiz & Castro, 2007; Thumar & Singh, 2009; Doukyu & Ogina, 2010). An organic solvent-tolerant, halophilic α-amylase has been purified from *Nesterenkonia* sp. (Shafiei *et al*., 2011). Fukushima and associates (2005) also reported an organic solvent-tolerant halophilic α-amylase from the extremely halophilic archaea, *Haloarcula* sp. Strain S-1. However, no such reports regarding the isolation of organic solvent α-amylases from plant sources have been made. In the present study, we report the stability of soybean α-amylase to various organic solvents. Besides, the considerable importance of this enzyme in detergent industries has led us to study the effect of some surfactants on the activity and stability of soybean α-amylase. Moreover, the possibility of reusing the enzyme for organic-solvent catalysis or detergent applications has been studied by immobilizing it on various matrices.

MATERIALS AND METHODS

Soybean seeds were procured from the local market. Sodium alginate, Maltose, Gelatin and 3,5-dinitrosalicylic acid (DNS) were purchased from Sigma Chemicals Co. (St. Loius, MO, USA). Soluble starch was obtained from Qualigens Fine Chemicals, Mumbai. All other reagents were analytical grade chemicals, either from BDH or E. Merck, India.

Isolation and purification of α-amylase. α-amylase was isolated from soybean (*Glycine max*) seeds, procured from the local market, and was purified to electrophoretic homogeneity as described previously (Prakash & Jaiswal, 2010).

α-amylase assay. α-amylase activity was estimated following the method as described by Bernfeld (1955).

Immobilization of α-amylase on various matrices. Immobilization of α-amylase on agarose, agar and gelatin matrices was carried out as described by Jaiswal and Prakash (2011a and 2011b).

Immobilized enzyme assay. For the assay of immobilized α-amylase, a desired number of immobilized chips containing 2.0–3.0 μg protein/chip) suspended in 0.5 mL assay buffer (Tris acetate, 150 mM, pH 5.5) was incubated with 0.5 mL of 1% potato starch solution at 27°C for 3 min and the reaction was stopped with 1.0 mL of 3,5-dinitrosalicylic acid followed by heating the reactants in a boiling water bath for 5 min and then cooling down to room temperature. After the addition of 10.0 mL of double distilled water, the amount of reducing sugar (maltose) produced was determined by spectrophotometer at 540 nm. In order to check the reusability, the chips were recovered from the reaction mixture, rinsed thoroughly with the buffer and stored in an extraction buffer (sodium acetate, 25 mM, pH 5.5) at 4°C.

✉e-mail: nive_june29@yahoo.co.in
Abbreviations: DNS, 3,5-dinitrosalicylic acid.

Protein estimation. Protein was estimated by the method of Lowry *et al.* (1951) with Folin-ciocalteau reagent calibrated with crystalline bovine serum albumin.

Effect of organic solvents on soluble enzyme. The activation/inhibition effect of different concentrations (10-50%, v/v) of various organic solvents (non-polar, polar aprotic, and polar protic) on the enzyme activity was tested by assaying the activity of the suitably diluted enzyme in their presence (indirect exposure). In order to explore the stability of the enzyme to the organic solvents, the enzyme was pre-incubated with different concentrations of organic solvents (direct exposure) for 30 and 60 min and then the residual activity of the exposed enzyme was determined.

Effect of surfactants on soluble enzyme. The indirect effect of various surfactants (sodium dodecyl sulphate, Triton X100 and Tween 20) on the activity of the suitably diluted enzyme was determined by varying their concentrations (10–50%, v/v) in the standard assay mixture and assaying the enzyme activity. Simultaneously, the suitably diluted enzyme was pre-incubated for 30 and 60 min with the varying concentration of the surfactants in order to determine the stability of the enzyme to the direct effects of these surfactants.

Effect of organic solvents and surfactants on immobilized enzyme. The solvent and surfactant, which showed an inhibition in the enzyme activity with soluble enzyme, were used to study their effects on the immobilized enzyme. The activity of the immobilized enzyme was assayed in the presence of varying concentrations (10–50% v/v) of these solvents/surfactants added in the standard assay mixture. The direct effect of the organic solvents/surfactants on the enzyme was explored by treating the immobilized α-amylase chips (2.0–3.0 μg protein/chip) with varying concentrations of these (in absence of substrate) for 30 min and then assaying for activity. The reusability of the immobilized chips was studied by assaying the activity of the same chips exposed to the reagents (at a concentration of 50% v/v for 30 min) with a time interval of 24 h between each activity determination. The used chips were rinsed again with sodium acetate buffer (25 mM, pH 5.5) 3–4 times and stored in the same buffer at 4°C for subsequent use.

RESULTS AND DISCUSSION

α-amylases have found potential applications in a wide number of industrial processes such as food, fermentation, textile, paper, detergent, pharmaceutical and fine chemical industries. However, given the advances in biotechnology, its application has widened in many fields such as clinical, medicinal and analytical chemistry as well as in starch saccharification and in the textile, food, brewing, and distilling industries (Gupta *et al.*, 2003; Pandey *et al.*, 2000, Arpana *et al.*, 2010). Here, we investigate the potential of the soybean α-amylase in detergent and pharmaceutical industries.

Effect of organic solvents on soluble enzyme

The impact of various organic solvents: non-polar (viz. cyclohexane, benzene, toluene and chloroform), aprotic polar (viz. dichloromethane, acetonitrile, acetone, dimethyl sulfoxide and dimethyl formamide) and protic polar (viz. methanol, ethanol, n-propanol, n-butanol and ethylene glycol) in the concentration range of 10–50% (v/v) added in the standard assay system was studied. Besides, the direct effect of these solvents on the enzyme was explored by treating the enzyme preparations with varying concentrations of organic solvents (in the absence of substrate) for 30 and 60 min and then assaying the activity.

Both the former and the latter case revealed a concentration dependent effect (either activation or inhibition) on soybean α-amylase activity with all the solvents studied. All the non-polar solvents used except chloroform showed an increase in the enzyme activity and also were quite stable (observed upon 30 and 60 min direct exposure of the enzyme to these). Chloroform showed a continuous and time-dependent decrease in enzyme activity

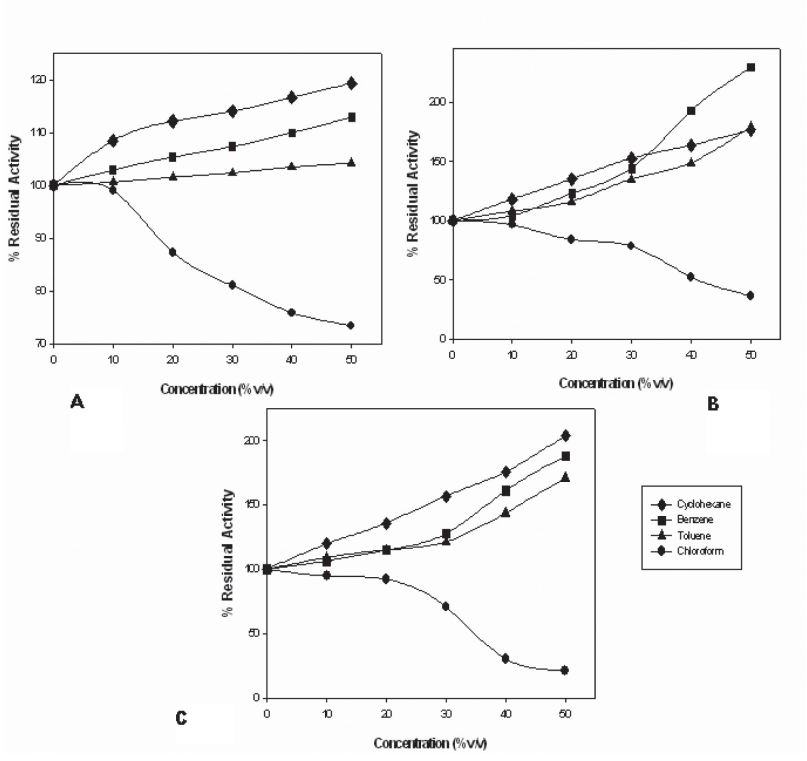

Figure 1. Effect of non-polar organic solvents on soybean α-amylase activity.
(**A**) Indirect exposure: Suitably diluted enzyme (13.0–15.0 U/mL, 4.0–5.0 μg protein/mL) was incubated with varying concentrations (10–50%, v/v) of solvents (in presence of substrate) added in the standard assay mixture and then assayed for activity. (**B**) Direct exposure (for 30 min): Suitably diluted enzyme (13.0–15.0 U/mL, 4.0–5.0 μg protein/mL) was pre-incubated for 30 min with varying concentrations (10–50%, v/v) of solvents (in absence of substrate) and then assayed for activity. (**C**) Direct exposure (60 min): Suitably diluted enzyme (13.0–15.0 U/mL, 4.0–5.0 μg protein/mL) was pre-incubated for 60 min with varying concentrations (10–50%, v/v) of solvents (in absence of substrate) and then assayed for activity.

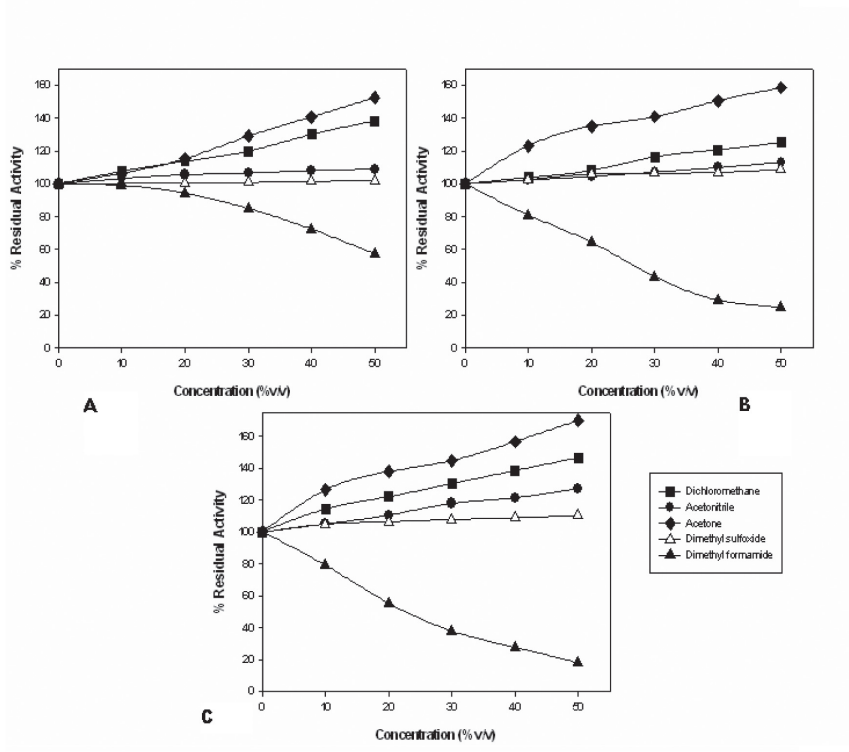

Figure 2. Effect of aprotic polar organic solvents on soybean α-amylase activity. (**A**) Indirect exposure: Suitably diluted enzyme (13.0–15.0 U/mL, 4.0–5.0 µg protein/mL) was incubated with varying concentrations (10–50%, v/v) of solvents added in the standard assay mixture and then assayed for activity. (**B**) Direct exposure (for 30 min): Suitably diluted enzyme (13.0–15.0 U/mL, 4.0–5.0 µg protein/mL) was pre-incubated for 30 min with varying concentrations (10–50%, v/v) of solvents (in absence of substrate) and then assayed for activity. (**C**) Direct exposure (for 60 min): Suitably diluted enzyme (13.0–15.0 U/mL, 4.0–5.0 µg protein/mL) was pre-incubated for 60 min with varying concentrations (10–50%, v/v) of solvents (in absence of substrate) and then assayed for activity.

with its increasing concentration (Fig. 1). The enzyme retained about 73% residual activity upon indirect exposure of the enzyme to this solvent. However, it showed only about 36 and 21% residual activity upon 30 and 60 min of direct exposure to chloroform, respectively. A similar trend of the concentration and time-dependent increase in the activity and stability of the enzyme upon indirect and direct exposure of the enzyme to aprotic polar solvents was found, except for dimethyl formamide, which showed a concentration and time-dependent inhibition in soybean α-amylase activity similar to chloroform (Fig. 2). The enzyme retained only about 25 and 18% residual activity upon 30 and 60 min of direct exposure to dimethyl formamide, respectively, which was about 57% upon indirect exposure to this.

The protic polar solvents also followed the same trend as non-polar and aprotic polar ones, except for n-butanol, which showed an inhibition of about 37% upon indirect exposure and 40% and 50% upon direct exposure of the enzyme to this solvent for 30 and 60

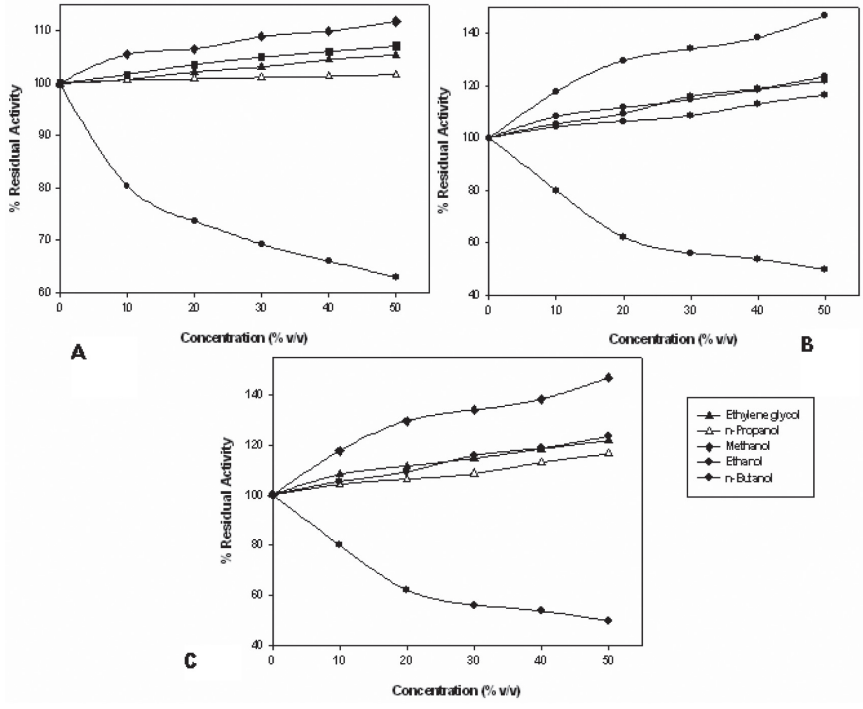

Figure 3. Effect of protic polar organic solvents on soybean α-amylase activity.
(**A**) Indirect exposure: Suitably diluted enzyme (13.0–15.0 U/mL, 4.0–5.0 µg protein/mL) was incubated with varying concentrations (10–50% v/v) of solvents added in the standard assay mixture and then assayed for activity. (**B**) Direct exposure (for 30 min): Suitably diluted enzyme (13.0–15.0 U/mL, 4.0–5.0 µg protein/mL) was pre-incubated for 30 min with varying concentrations (10–50%, v/v) of solvents (in absence of substrate) and then assayed for activity. (**C**) Direct exposure (for 60 min): Suitably diluted enzyme (13.0–15.0 U/mL, 4.0–5.0 µg protein/mL) was pre-incubated for 60 min with varying concentrations (10–50%, v/v) of solvents (in absence of substrate) and then assayed for activity.

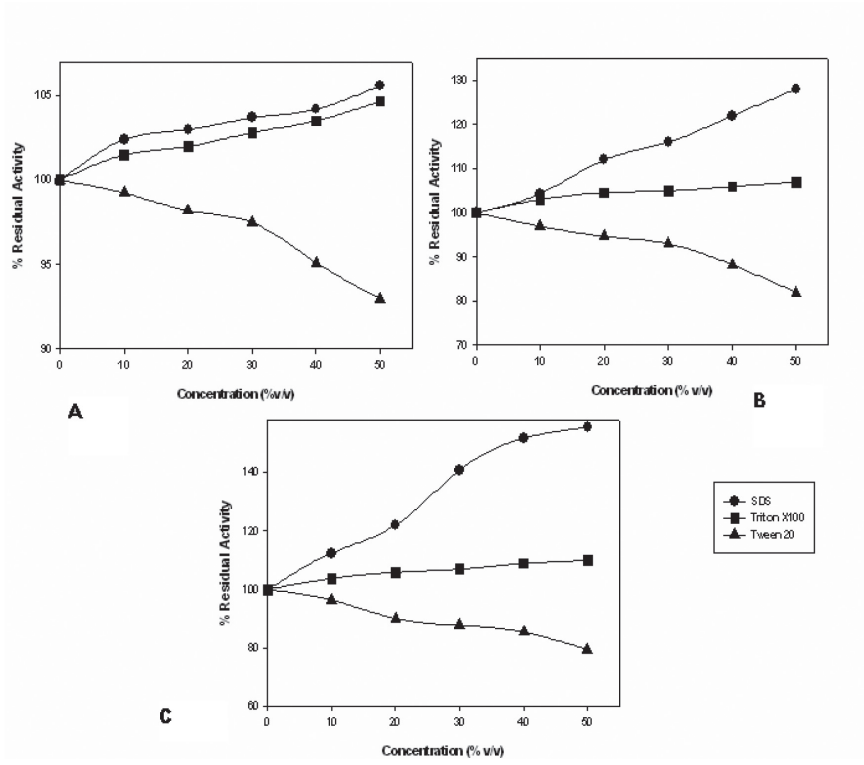

Figure 4. Effect of surfactants on soybean α-amylase activity.
(**A**) Indirect exposure: Suitably diluted enzyme (13.0–15.0 U/mL, 4.0–5.0 µg protein/mL) was incubated in varying concentrations (10–50%, v/v) of surfactants added in the standard assay mixture and then assayed for activity. (**B**) Direct exposure (for 30 min): Suitably diluted enzyme (13.0–15.0 U/mL, 4.0–5.0 µg protein/mL) was pre-incubated for 30 min in varying concentrations (10–50%, v/v) of surfactants (in absence of substrate) and then assayed for activity. (**C**) Direct exposure (for 60 min): Suitably diluted enzyme (13.0–15.0 U/mL, 4.0–5.0 µg protein/mL) was pre-incubated for 60 min in varying concentrations (10–50%, v/v) of surfactants (in absence of substrate) and then assayed for activity.

min respectively (Fig. 3). Fukushima and coworkers (2005) found the halophilic α-amylase (isolated from a Haloarchaeon, *Haloarcula* sp. Strain S-1) to be active and stable in benzene, toluene, and chloroform. However, no activity was detected in the presence of ethanol and acetone. Shafiei and associates (2011) found a remarkable stability of α-amylase (isolated from *Nsterenkonia* sp.) towards organic solvents like cyclohexane, benzene, toluene, and chloroform.

Thus, it was observed that the enzyme showed a remarkable enhancement in its activity and stability upon the exposure to the organic solvents (both indirect and direct) studied here except for few. This stability of the enzyme against these solvents may be due to the rigid enzyme structure or its lowered water requirement. These solvent stable amylases could be potentially useful in the pharmaceutical and fine-chemical industries.

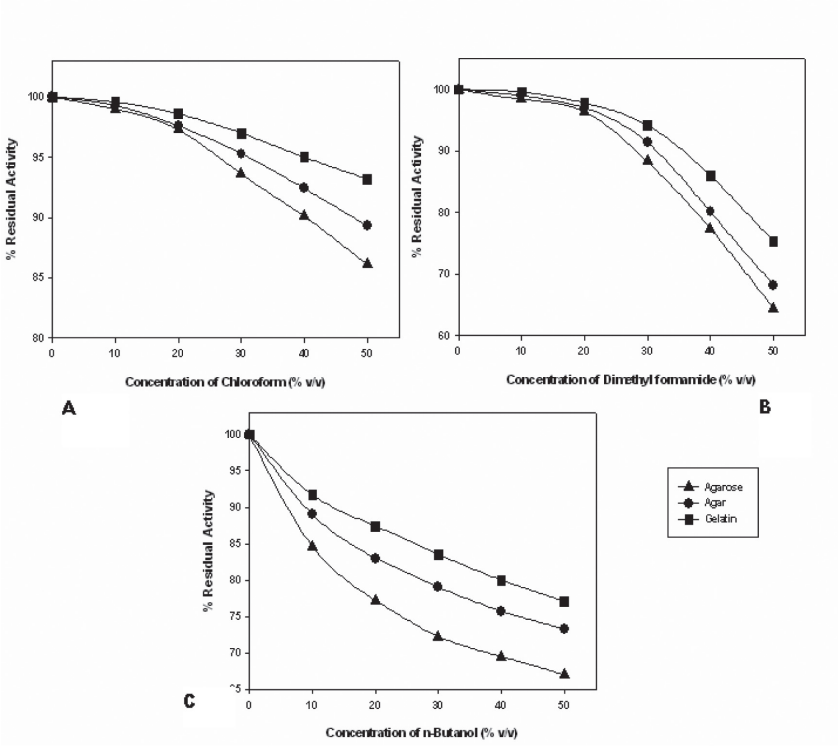

Figure 5. Effect of various organic solvents.
(**A**) Chloroform, (**B**) Dimethyl formamide, (**C**) n-Butanol) on agarose, agar and gelatin immobilized α-amylase activity. Two immobilized chips (2.0–3.0 µg protein/chip) were incubated with varying concentrations (10–50% v/v) of chloroform added in the standard assay mixture and then assayed for activity.

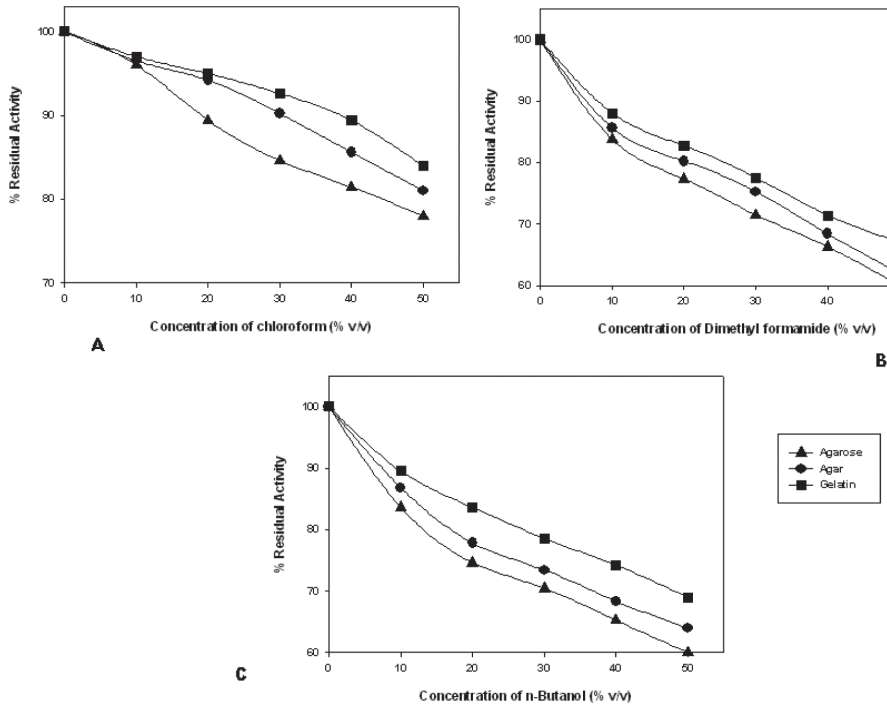

Figure 6. Effect of direct exposure (for 30 min) of various organic solvents.
(**A**) Chloroform, (**B**) Dimethyl formamide, (**C**) n-Butanol) on agarose, agar, and gelatin immobilized α-amylase activity. Two immobilized chips (2.0–3.0 µg protein/chip) were pre-incubated with varying concentrations (10–50%, v/v) of chloroform for 15 min (in absence of starch) and then assayed for activity.

Effect of surfactants on soluble enzyme

The impact of varying concentration of surfactants; SDS (in % w/v), Triton X100 and Tween 20 (both in % v/v) on the enzyme activity both upon indirect and direct exposures (30 and 60 min) of enzyme to these is presented in Fig. 4. As observed from the results, there was a continuing increase in the enzyme activity for SDS and Triton X100 both upon indirect and direct exposures (for 30 and 60 min). Similar activating effect on the enzyme activity with SDS was observed by de Oliviera and associates (2010). Tween 20 showed a concentration dependent decrease in the enzyme activity but the enzyme still retained about 93%, 82% and 79% residual activity upon indirect and direct exposure for 30 and 60 min, respectively. α-amylase isolated from a moderately halophilic *Nesterenkonia* sp. Strain F showed a remarkable stability towards surfactants like Tween 20 and Triton X100 (Shafiei *et al.*, 2011).

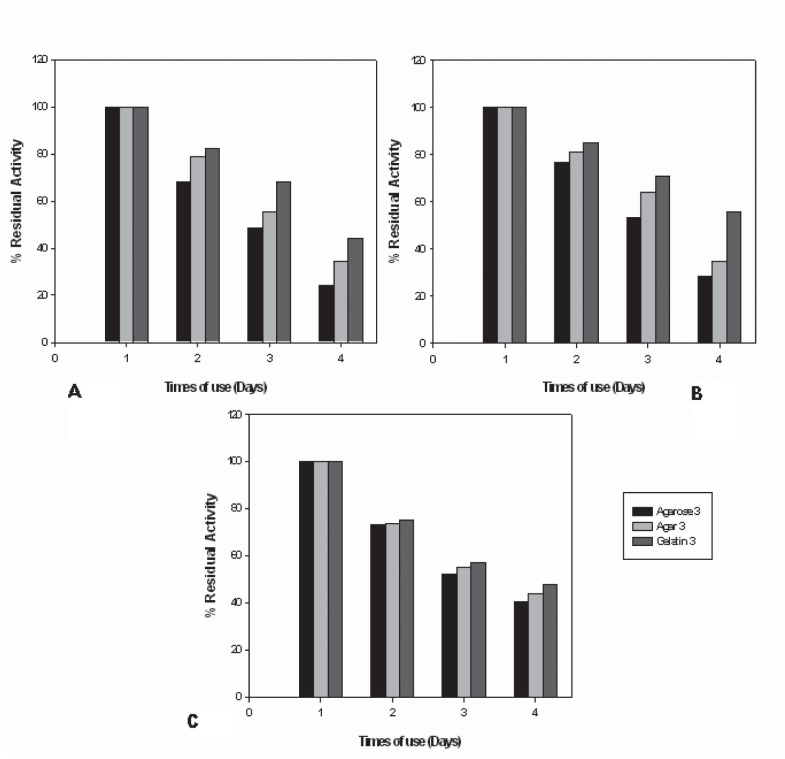

Figure 7. Bar representation of reusability of agarose, agar, and gelatin immobilized α-amylase after direct exposure (for 30 min) to:
(**A**) Chloroform, (**B**) Dimethyl formamide, (**C**) n-Butanol (50%, v/v). Two immobilized chips (2.0–3.0 µg protein/chip) exposed to chloroform (50%, v/v) for 30 min and kept at 4°C in Tris acetate buffer (150 mM, pH 5.5) were assayed for the activity on the respective day. The residual activity left after direct exposure for 30 min was taken as 100% and the residual activities after every 24 h of reuse was calculated accordingly.

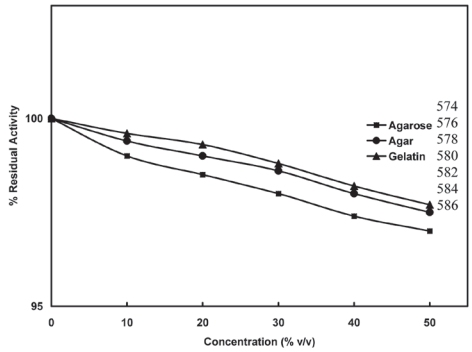

Figure 8. Effect of Tween 20 on agarose, agar, and gelatin immobilized α-amylase activity.
Two immobilized chips (2.0–3.0 µg protein/chip) were incubated with varying concentrations (10–50% v/v) of Tween 20 added in the standard assay mixture and then assayed for activity.

Effect of organic solvents and surfactants on immobilized enzyme

The impact of some organic solvents like chloroform, dimethylformamide and n-butanol (which showed an inhibition in the activity of soluble enzyme, (as descried above) on the immobilized system was studied in the concentration range of 10–50% (v/v) added in the standard assay system. The results revealed a concentration-dependent effect of the solvents on the immobilized chips (Fig. 5) similar to the case with soluble enzyme. The effect of chloroform (50% v/v) on soluble enzyme showed a residual activity of about 73%, while it was 86%, 89% and 93% in case of agarose, agar, and gelatin immobilized enzyme, respectively. Similarly, dimethyl formamide and n-butanol with soluble enzyme showed a residual activity of about 57% and 63%, respectively. However, the agarose, agar and gelatin immobilized enzyme retained an activity of about 64%, 68% and 75% (in case of dimethyl formamide) and about 68%, 73% and 77% (in case of n-butanol), respectively.

When the immobilized enzyme was first exposed to varying concentrations of organic solvents for 30 min and then assayed, it was noticed that the immobilized enzyme was fairly stable to the direct exposure to organic solvents (in the absence of substrate; Fig. 6). The immobilized enzyme upon an exposure of 30 min to chloroform showed a residual activity of 78%, 81% and 84% (with agar, agarose and gelatin). Similarly, the residual activity of the enzyme immobilized in agarose, agar, and gelatin upon the exposure to dimethyl formamide was 60%, 62%, and 67%, respectively and to that of n-butanol was 60%, 64% and 69%, respectively. Besides, the immobilized enzyme after the exposure to these solvents (at 50% v/v) for 30 min was found reusable for upto four times showing a decline in its activity after every time of use (Fig. 7).

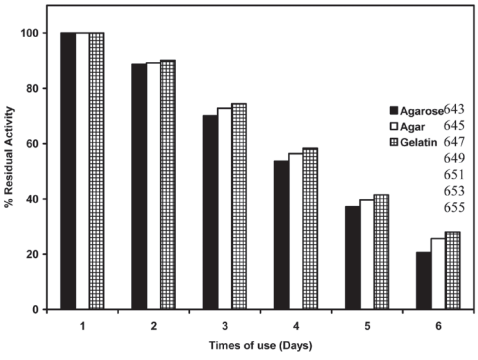

Figure 9. Bar representation of reusability of agarose, agar, and gelatin immobilized α-amylase after direct exposure (for 30 min) to Tween 20 (50% v/v).
Two immobilized chips (2.0–3.0 µg protein/chip) exposed to Tween 20 (50% v/v) for 30 min and kept at 4°C in Tris acetate buffer (150 mM, pH 5.5) were assayed for the activity on the respective day. The residual activity left after direct exposure to Tween 20 for 30 min was taken as 100% and the residual activities after every 24 h of reuse was calculated accordingly.

The effect of the surfactant, Tween 20 (as it showed an inhibition in case of soluble enzyme as already described in Materials and Methods section), on the immobilized system was studied in the concentration range of 10–50% (v/v) (Fig. 8). The enzyme retained more or less about complete activity as compared to about 93% of residual activity found in case of soluble enzyme. Upon 30 min of direct exposure of the immobilized chips to varying concentration of these surfactant, it was found that agarose, agar, and gelatin immobilized chips retained about 86%, 90% and 93% activity, respectively. Moreover, the exposed immobilized chips (for 30 min) to this surfactant showed reusability for upto six times (Fig. 9).

CONCLUSION

The good stability of soybean α-amylase to the organic solvents facilitates the remarkable and significant use of the enzyme in non-aqueous organic media, organic synthesis and chiral resolution. The enzyme was significantly more stable even in the presence of surfactants. Thus, it can be stated that the surfactants were not harmful to the structural integrity of the enzyme and thus the enzyme can work efficiently in detergent industries. Upon the comparison of the behaviour of soluble and immobilized enzyme in some organic solvents as well as surfactant tested here, it can be suggested that the organic solvents/surfactants are much more deleterious to soluble α-amylase than the immobilized enzyme. Thus, the immobilization imparted the stability of the enzyme for use in organic media as well in presence of surfactants. This stability could be an outcome of the interplay of water activity and support. Moreover, the reusability of the enzyme after immobilization upon the exposure to these suggests that besides imparting stability, it also keeps the enzyme active for a longer duration of time.

REFERENCES

Bernfeld P 1(955) Amylase alpha and beta. In *Methods in Enzymology*. Colowick SP, Kaplan NO, eds, pp 149–152. Academic Press, New York, USA.

Carrea G, Ottolina, Riva S (2000) Properties and synthetic applications of enzymes in organic solvents. *Angew Chem* **33**: 2226–2254.

de Oliveira AN, de Oliveira LA, Andrade JS (2010) Partial characterization of amylases of two indigenous central amazonian rhizobia strains. *Braz Arch Biol Technol* **53**: 35–45.

Doukyu N, Ogino H (2010) Organic solvent-tolerant enzymes. *Biochem Eng J* **48**: 270–282.

Fukushima T, Mizuki T, Echigo A, Inoue A, Usami R (2005) Organic solvent tolerance of halophilic *a*-amylase from a Haloarchaeon, *Haloarcula* sp. strain S-1. *Extremophiles* **9**: 85–89.

Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003) Microbial α-amylases: a biotechnological perspective. *Process Biochem.* **38**: 1599–1616.

Jaiswal N, Prakash O (2011 a) Immobilization of a thermostable α-amylase on agarose and agar matrices and its application in starch stain removal. *World Appl Sci J* **13**: 572–577.

Jaiswal N, Prakash O (2011 b) Immobilization of soybean α-amylase on gelatin and its application as a detergent additive. *Asian J Biochem* **6**: 337–346.

Klibanov AM (1989) Enzymatic catalysis in anhydrous organic solvents. *Trends Biochem Sci* **14**: 141–144.

Koskinen AMP, Klibanov AM, eds, (1996) *Enzymatic reactions in organic media*. Blackie, London.

Kumari A, Singh VK, Fitter J, Polen T, Kayastha AM (2010) α-amylases from germinating soybean (Glycine max) seeds- Purification, characterization and sequential similarity of conserved and catalytic amino acid residues. *Phytochem* **71**: 1657–1666.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature* **227**: 680–685.

Lowry OH, Rosebrough NJ, Farr AL, Randall PJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* **193**: 265–275.

Ogino H, Nakagawa S, Shinya K, Muto T, Fujimura N, Yasuda M, Ishikawa H (2000) Purification and characterization of organic solvent-stable lipase from organic solvent-tolerant *Pseudomonas aeruginosa* LST-03. *J Biosci Bioeng* **89**: 451–457.

Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R (2000) Advances in microbial amylases. *Biotechnol Appl Biochem* **31**: 135–152.

Prakash O, Jaiswal N (2010) A highly efficient and thermostable α-amylase from soya bean seeds. *Biotechnol Appl Biochem* **57**: 105–110.

Ruiz DM, De Castro RE (2007) Effect of organic solvents on the activity and stability of an extracellular protease secreted by the haloalkaliphilic archaeon *Natrialba magadii*. *J Ind Microbiol Biotechnol* **34**: 111–115.

Sellek GA, Chaudhuri JB (1999) Biocatalysis in organic media using enzymes from extremophiles. *Enzy Microb Technol* **25**: 471–482.

Serdakowsli AL, Dordick JS (2008) Enzyme activation for organic solvents made easy. *Trends Biotechnol* **26**: 48–54.

Shafiei M, Ziaee AA, Amoozegar MA (2011) Purification and characterization of an organic-solvent-tolerant halophilic α-amylase from the moderately halophilic *Nesterenkonia* sp. strain F. *J Ind Microbiol Biotechnol* **38**: 275–281.

Thumar JT, Singh SP (2009) Organic solvent tolerance of an alkaline protease from salt-tolerant alkaliphilic *Streptomyces clavuligerus* strain Mit-1. *J Ind Microbiol Biotechnol* **36**: 211–218.

Zaks A, Klibanov AM (1984) Enzymatic catalysis in organic media at 100°C. *Science* **224**: 1249–1251.