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BIOSYNTHESIS, PURIFICATION AND CHARACTERIZATION OF COMMERCIAL ENZYME BY *PENICILLIUM EXPANSUM* LINK

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Abstract

Ever growing biotechnological industry has motivated the research towards the comprehensive survey of microorganisms, which could be used in extreme conditions of industry. In the present work optimization parameters in submerged fermentation, purification and characterization of invertase from *Penicillium expansum* Link using agricultural wastes (sunflower waste, cotton stalk and rice husk) as well as agro-industrial wastes (date syrup and molasses) as sources of carbon. Maximum production of invertase (7.03 U/mL) was observed when the strain was grown on culture medium (CM1) containing yeast extract as a source of nitrogen, date syrup as a source of carbon after 48 h of incubation at initial pH 5.0, temperature 35ºC, inoculum size of $6x10^6$ conidia in 50 mL of culture medium and agitation rate of 150 rev/min. After optimization the enzyme was also purified partially and then characterized. Kinetic constants (K_m 2.57 mM and V_{max} 178.6 U/mL/min) were determined by Lineweaver-Burk Plot and molecular mass (110 kDa) by 10% SDS-PAGE. Invertase showed maximum activity at pH 5.5 (128.7 U/mL) and at the temperature of 60ºC (114.6 U/mL). $BaCl_2$ (21.9%), $MgSO_4$ (42.6%), $MnCl_2$ (46.8%) and EDTA (8.3%) enhanced the relative activity of enzyme while $HgCl_2$ (-90.9%), $CuSO_4$ (-82.3%) and $CuCl_2$ (-78.7%) were proved inhibitors.

Key words: Invertase, Submerged fermentation, Commercial enzyme.

Introduction

The modern biotechnological setup due to increasing demand of enzymes has motivated the need for enlarged survey of microorganisms surviving and producing enzyme in extreme conditions (Mamma *et al.,* 2008). For the production of large quantities of enzymes filamentous fungi have biotechnological importance (Ahmed *et al.,* 2011; 2014).

Invertase (E. C. 3.2.1.26), splits sucrose into glucose and fructose. It is one of the most widely used enzymes by food industry in making chocolate covered cherries. This enzyme is also used in paper industry and to make artificial honey in which it contributes to anti-bacterial properties (Phadtare *et al.*, 2004; Kotwal & Shankar, 2009; Safarik *et al.*, 2009; Kulshrestha, 2013).

In the present work specific interest has been focused on agriculture waste like sunflower cotton stalk rice husk, date syrup, and molasses because they are usually related with pollution. Being the cost effective sources of carbon agricultural wastes have a potential for conversion into useful products (Mamma *et al.,* 2008). In this work the secretion of invertase by *Penicillium expansum* Link in submerged fermentation was carried out. After optimization the enzyme was also purified and characterized.

Materials and Methods

Strain and fermentation medium: Strain of *Penicillium expansum* Link (IBGE 06) was obtained from the Institute of Biotechnology and Genetic Engineering, University of Sindh Jamshoro and culture were maintained as followed by Dahot (1986). In the present study slants of 4 days old were used for inoculation. Number of conidia of each fungus was counted by haemocytometer. Spore suspension was maintained around $4x10^6$ conidia. Each agricultural waste (cotton stalk, sunflower waste and rice husk) were hydrolyzed by following Dahot & Abro (1994). An agricultural waste hydrolysed with sulphuric acid produces a variety of sugars and their degradation products (Ahmed *et al*., 2011). Protein in the enzyme sample was determined by the method of Lowry *et al*. (1951) and invertase activity was determined by following Akgol *et al.* (2001). One unit of invertase activity is the amount of enzyme, which releases 1 mg of inverted sugar in 5 min at 35ºC and pH 5.5.

Optimization of enzyme production parameters: All experiments were done in such a way that the parameter optimized in one experiment was fixed in the next experiments for the production of enzyme. First of all the most suitable culture medium was determined. For invertase production 50 mL of following culture media were used in 250 mL flask having composition (in g/L).

CM1: Dextrose 10, peptone 5, epsom salt 5, KH_2PO_4 5, common salt 2.5, ferrous sulphate hepta hydrate 0.01, $ZnSO_4.7H_2O$ 0.002, $MnSO_4.H_2O$ 0.001 and thiamine hydrochloride 0.001 (Burrel *et al.,*1966).

CM2: Yeast extract 10, peptone 20 and sucrose 20. (Dworschock & Wickerham, 1961).

CM3: Yeast extract 20, peptone 40, sucrose 20, $KH_2(PO_4)_2$ and epsom salt 1 (Souza *et al*., 2007).

CM4: $NaNO_3$ 3, KCl 0.5, epsom salt 0.5, ferrous sulphate hepta hydrate 0.01, K_2HPO_4 1, Sucrose 30 (Almeida *et al*., 2005).

CM5: Sucrose 40, corn steep liquor 30, $NaNO_3$ 3, KH_2PO_4 0.5, epsom salt 0.05, $CaCO_3$ 2.5 (Poonawalla *et al*., 1965).

After the determination of the most suitable culture medium, incubation time period, the most suitable carbon source, nitrogen source, incubation temperature, initial pH of medium, inoculum size and agitation rate for maximum production of invertase were determined in a sequence

Purification and characterization of enzyme: After optimization of all parameters, purification and characterization of invertase was done. In the first step varying amounts (20-85%) of ammonium sulphate were added to cell free supernatant and then precipitates were obtained by spinning at 20,000 x g for 20 minutes. Precipitates were then dissolved in 0.5 M tris-hydrochloric acid buffer at pH 7.5 and dialyzed. The crude enzyme was loaded onto a DEAE-cellulose column (1.0 x 10.0 cm), which was equilibrated with 100 mM of tris-HCl buffer at pH 7.5. With the linear salt gradient (sodium chloride, 0-1 M) the enzyme was eluted in the same buffer and 2.9 mL of fractions were obtained at 20 mL per hour flow rate at 4°C. The collected fractions were analyzed for invertase activity. Only those fractions having high enzyme activities were pooled, dialyzed and examined on SDS-PAGE. Effects of various additives, temperature and pH optima of invertase were examined (Akgol *et al*., 2001). Molecular mass of purified enzyme was determined by 10% SDS-PAGE and kinetic constants K_m and V_{max} were calculated with the help of Lineweaver-Burk Plot (Lineweaver & Burk, 1934).

Results and Discussions

Effect of culture media: Effects of various culture media on invertase production by *P. expansum* after 24 h, at 30°C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are plotted (Fig. 1). The strain was grown on five different culture media CM1, CM2, CM3, CM4 and CM5. It was capable of growing well on all types of culture media but production of invertase was maximum (1.57 U/mL) on culture medium CM1, which was selected for the following study.

Selection of the most suitable culture media has the deep effect on enzyme production. Many researchers all over the world have reported different culture media for maximum invertase production (Dworschack & Wickerham, 1961; Poonawalla *et al*., 1965; Herwig *et al.,* 2001; Almeida *et al*., 2005; Souza *et al*., 2007).

Effect of incubation time period: The effects of incubation time periods on invertase production by *P. expansum* IBGE 06 in CM1 at temperature 30ºC, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are presented (Fig. 2). Invertase activity was measured at regular interval of 24 h and it was found that the maximum activity (2.46 U/mL) was observed after 48 h of incubation. On prolonged incubation enzyme activity was decreased, which might be due to denaturing of enzyme or synthesis of inhibiting metabolite (Mamma *et al*., 2008). Incubation time period of 48 h was also reported for invertase production from *Saccharomyces cerevisiae* (Mizunaga *et al*., 1981).

Effect of carbon sources: The effects of various carbon sources on invertase production by *P. expansum* after 48 h in CM1 at temperature 30°C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are exhibited (Fig. 3). It was observed that invertase activities were lower in case of 0.3*N* sulphuric acid hydrolysed agriculture waste (1.64, 1.31 and 1.48 U/mL for cotton stalk, sunflower waste and rice husk respectively) and 0.5% of molasses and date syrup (2.42 U/mL and 2.17 U/mL respectively). Invertase activities were closed to or higher than control, glucose (2.46 U/mL) when 0.6*N* sulphuric acid hydrolysed agriculture waste (2.62, 2.73 and 2.18 U/mL for cotton stalk, sunflower waste and rice husk respectively) and 1% of molasses (3.25 U/mL) and date syrup (2.94 U/mL) were used. Sugarcane bagasse was reported by Guimarães *et al*., (2007) as the appropriate carbon source for invertase production by *Aspergillus niveus.*

Effect of nitrogen sources: The effects of various nitrogen sources on invertase production by *P. expansum* IBGE 06 after 48 h in CM1 containing molasses as carbon source at 30°C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are shown (Fig. 4). The strain showed the capability of utilizing well all types of nitrogen sources but yeast extract was found to be the best (3.13 U/mL in 0.25% and 3.78 U/mL in 0.50%). Yeast extract was also reported as the best nitrogen source for *Saccharomyces cerevisiae* (Dworschack & Wickerham, 1961) and *Aspergillus ochraceus* (Guimarães *et al*., 2007). Surprisingly, the strain *P. expansum* showed high values of invertase activities with urea (2.18 U/mL in 0.25% and 3.01 U/mL in 0.5%), which might be due to urease production by the strain (Egorov *et al*., 2000; Hussain *et al.,* 2010).

Effect of temperature: The effects of incubation temperatures on invertase production by *P. expansum* after 48 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, at initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are presented (Fig. 5). The fermentation medium was incubated at a range of temperatures 20-70°C. Invertase activity was the highest (4.53 U/mL) about 35°C. Similar optimum temperature was reported for *Aspergillus niger* by Ashokumar *et al*., (2001). Interestingly the strain showed thermo stability up to 60°C (0.69 U/mL), which is a requirement for industrial use of a microorganism (Mamma *et al*., 2008).

Effect of initial pH: The effects of initial pH of fermentation medium on invertase production by *P. expansum* after 48 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, temperature 35°C, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are plotted (Fig. 6). The range of pH (4.0 to 9.0) was studied and found that initial pH of 5.0 would be the best for optimum enzyme production (5.41 U/mL). Similar optimum pH was reported by Uma *et al.* (2010) from *Aspergillus flavus*.

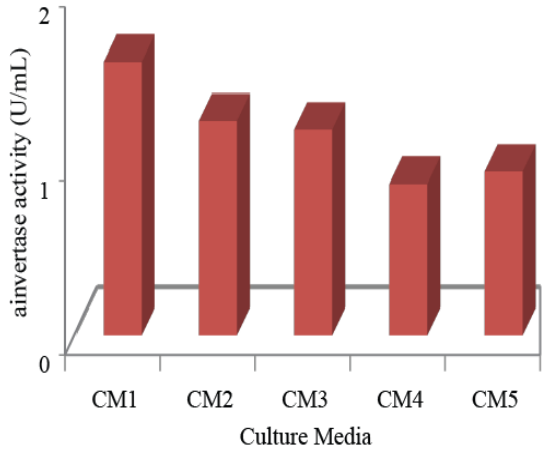

Fig. 1. Effects of various culture media on invertase production by *Penicilium expansum* after 24 h, at 30ºC, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min.

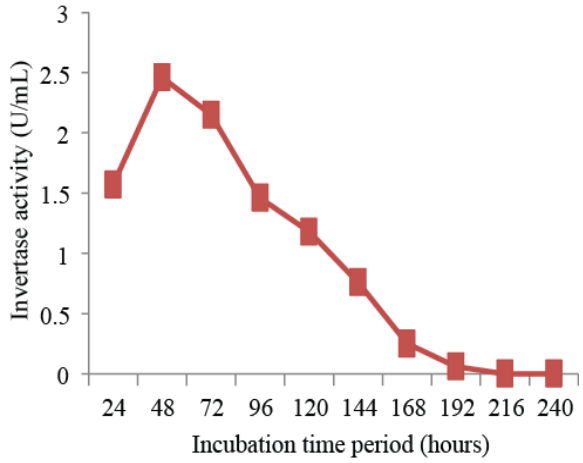

Fig. 2. Effects of incubation time periods on invertase production by *P. expansum* in CM1 at 30ºC, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min.

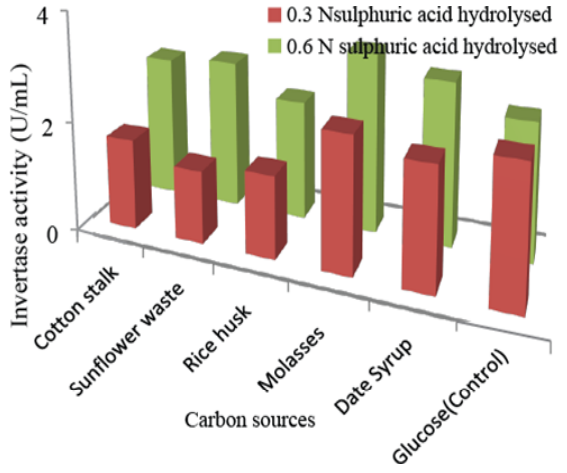

Fig. 3. Effects of various carbon sources on invertase production by *P.expansum* after 48 h in CM1 at 30ºC, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min.

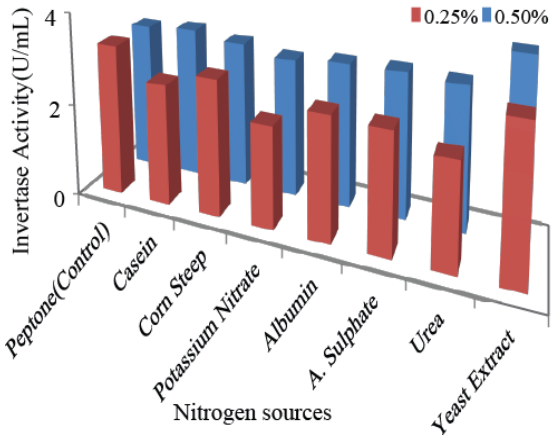

Fig. 4. Effects of various nitrogen sources on invertase production by *P. expansum* after 48 h in CM1 containing molasses as carbon source at 30ºC, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min.

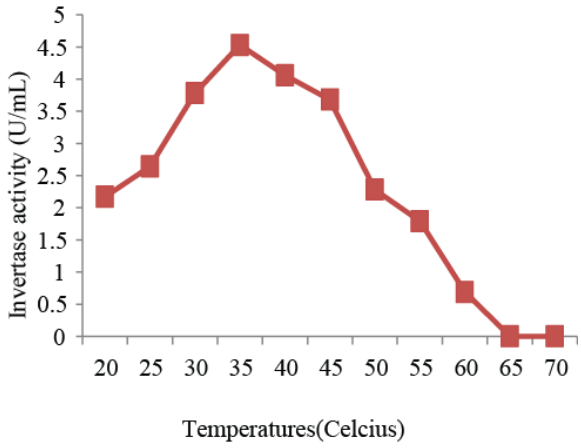

Fig. 5. Effects of incubation temperature on invertase production by *P. expansum* after 48 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, at initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min.

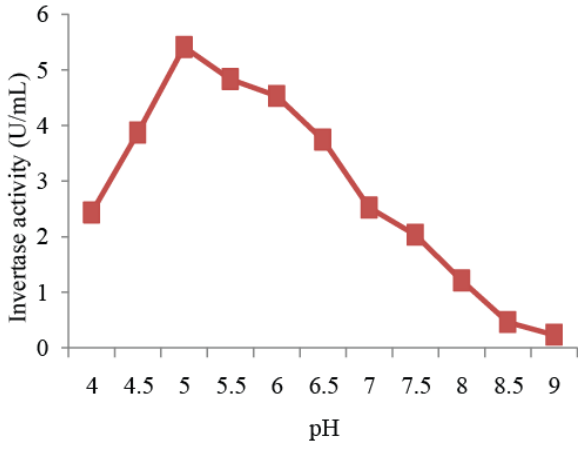

Fig. 6. Effects of initial pH of fermentation medium on invertase production by *P. expansum* after 48 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, at 35ºC, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min.

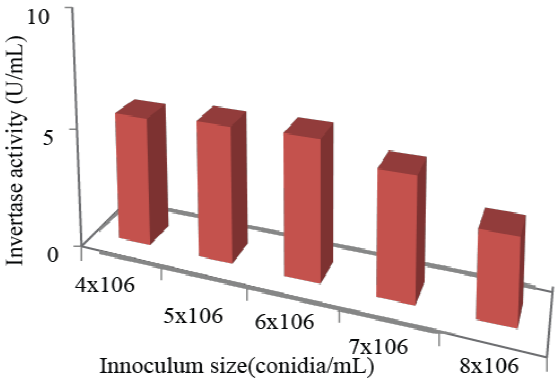

Fig. 7. Effects of inoculum sizes on invertase production by *P. expansum* after 48 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, at 35ºC, initial pH 5.0 and agitation rate 50 rev/min.

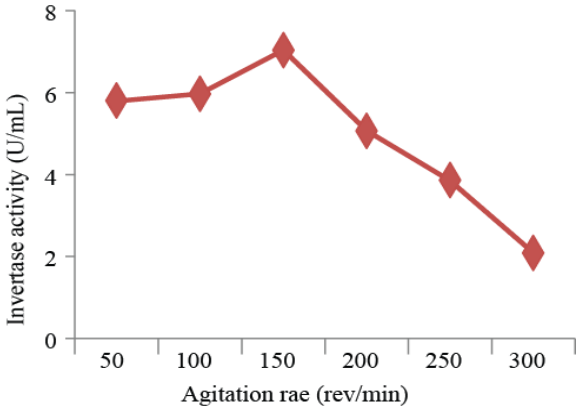

Fig. 8. Effects of agitation rates on invertase production by *P. expansum* after 48 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, at 35ºC, initial pH 5.0 and inoculum size $6x10^6$ conidia.

Effect of inoculum size: Effects of inoculum sizes on invertase production by *P. expansum* after 48 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, 35°C, at initial pH 5.0 and agitation rate 50 rev/min are shown (Fig. 7). Flasks were added with $4x10^6$-$8x10^6$ conidia and maximum invertase activity (5.79 U/mL) was observed when $6x10^6$ conidia were added to the medium. Literature Survey revealed that researchers used varying inoculum sizes (Dahot, 1986; Guimarães *et al*., 2007; Mamma *et al*., 2008; Ahmed *et al*., 2011).

Effect of agitation rate: The effects of agitation rates on invertase production by *P. expansum* after 48 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, at 35°C, initial pH 5.0 and inoculum size $6x10^6$ conidia are presented (Fig. 8). The fermentation medium was agitated at 50, 100, 150, 200, 250 and 300 rev/min. Invertase activity was maximum (7.03 U/mL) at 150 rev/min. Various agitation rates (100-200 rev/min) have been reported for enzymes production by different microorganisms (Dahot, 1986; Quiroga *et al*., 1995; L'Hocine *et al*., 2000; Rubio *et al*., 2002; Bhatti *et al*., 2006).

Purification and characterization of enzyme:

Purification: The extracellular invertase from *Penicillium expansum* was purified and summary of steps is given in Table 1.

Chan *et al*. (1991) reported 75% recovery of yeast's invertase with nine fold purification using 0.05*M* tris HCl buffer containing 0.5*M* sodium chloride at pH 7. Bhatti *et al*. (2006) purified invertase from *Fusarium solani* to homogeinity by ammonium sulfate precipitation and column chromatography *i.e.,* DEAE-cellulose and Sephadex G-200.

Characterization of purified Invertase

Determination of kinetic parameters (K_m & V_{max}): The Michaelis-Menten Constant (K_m) is defined as the substrate concentration at half the maximum velocity V_{max}. Both kinetic parameters (K_m 2.57 mM and V_{max} 178.6 U/mL/min) of purified invertase from *Penicillium expansum* were determined by Lineweaver-Burk Plot (Fig. 9) for sucrose. Varying Km and V_{max} values were reported in Literature. L'Hocine *et al*. (2000) reported Km (44.38 mM) and V_{max} (1030 mmol/mL/min) values of invertase from *Aspergillus niger*. Guimarães *et al*. (2007) calculated K_m (7.37, 13.4 and 2.66 mM) and V_{max} (22.39, 42.13 and 3.14 U/mg) values of invertase from *Aspergillus ochraceus*. Bhatii *et al.* (2006) reported K_m (3.57 mM) of invertase from *Fusarium solani*.

Effect of pH and temperature on purified invertase activity: The effects pH (Fig. 10) and temperature (Fig. 11) on purified enzyme were examined and it was found that invrtase showed maximum activity at pH 5.5 (128.7 U/mL) and 60°C (114.6 U/mL). Above and below these conditions there is a reduction in invertase activity. The results are similar to that reported for *Aspergillus niger* by L'Hocine *et al*. (2000) while Bhatti *et al*. (2006) described that invertase produced by *Fusarium solani* the optimum pH and temperature were 2.6 and 50°C respectively.

Effects of some additives (compounds) on invertase acivity: Various compounds such as $BaCl_2$, $CuCl_2$, $MgCl_2$, KCl, NaCl, NH_4Cl, EDTA, $MgSO_4$, $MnCl_2$, $HgCl_2$, $CuSO_4$, $ZnSO_4$, $CaCl_2$, , $CoCl_2$, $AgNO_3$, $NiSO_4$ and $FeSO_4$ (all 1mM) were incubated with purified enzyme at 30°C for 30 minutes and then invertase activities were determined. Table 2 shows the results in which $BaCl_2$ (21.9%), $MgSO_4$ (42.6%), $MnCl_2$ (46.8%) and EDTA (8.3%) enhanced the relative activity of enzyme while $HgCl_2$ (-90.9%), $CuSO_4$ (-82.3%) and $CuCl_2$ (-78.7%) were proved inhibitors. Similar reults were reported by Guimarães *et al*. (2007) for invertase of *Aspergillus ochraceus*.

Determination of molecular mass: Molecular mass of purified enzymes are reported in kilo Dalton (1 amu = 1 Dalton), which is usually determined by SDS-PAGE (Rubio *et al*., 2002). The purity and molecular mass determination of invertase from *Penicillium expansum* by subjecting to 10% SDS-PAGE (Fig. 12). [Red: under reduced dissociating/denaturing conditions. Lane M: molecular weight marker (Novagen, USA)]. It shows the enzyme is almost purified and molecular mass was found 110 kDa. Milintawisamai *et al.* (2007) also found the molecular mass of extracellular invertase from *C. humicolus* as 110 kDa. In *Xanthophyllomyces dendrorhous* invertase was found to be as glycoprotein with molecular mass of 160 kDa (Linde *et al.,* 2009).

Table 1. Purification steps of extra cellular invertase by *Penicillium expansum*.

Treatment	Volume	Total activity	Total protein	Specific activity	Yield	Fold purification
	(mL)	(U)	(mg)	(U/mg)	(%)	
Crude enzyme	500	6986	216.4	32.28	100	1
A. sulphate (80%) treated	39	2839	62.5	45.4	40.6	1.4
DEAE-cellulose	6.4	1785	8.3	215.1	25.5	6.7

Table 2. Effects of additives on stability of purified invertase by *Penicillium expansum*.

S. No.	Addition	Relative activity (%)	S. No.	Addition	Relative activity (%)
1.	None (Control)	100	10.	$MgSO_4$	142.6
2.	$MnCl_2$	146.8	11.	$HgCl_2$	8.9
3.	$BaCl_2$	121.9	12.	$CuSO_4$	17.7
4.	$CuCl_2$	21.3	13.	$ZnSO_4$	96.9
5.	$MgCl_2$	105.2	14.	$CaCl_2$	89.7
6.	KCl	97.1	15.	$CoCl_2$	101.5
7.	NaCl	101.4	16.	$AgNO_3$	91.4
8.	NH_4Cl	74.5	17.	$NiSO_4$	92.1
9.	EDTA	108.3	18.	$FeSO_4$	97.5

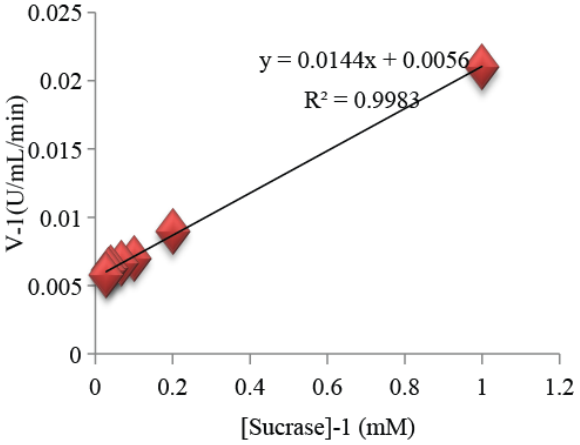

Fig. 9. Lineveaver-Burk plot for kinetic constants of invertase from *Penicillium expansum*.

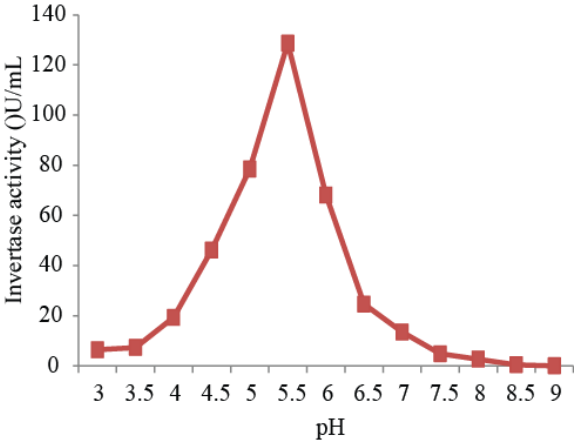

Fig. 10. Effects of pH on purified invertase from *P. expansum*.

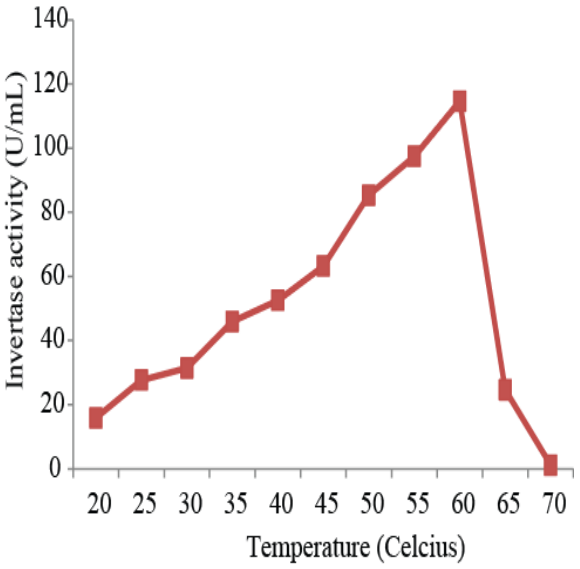

Fig. 11. Effects of temperatures on purified invertase from *P. expansum*.

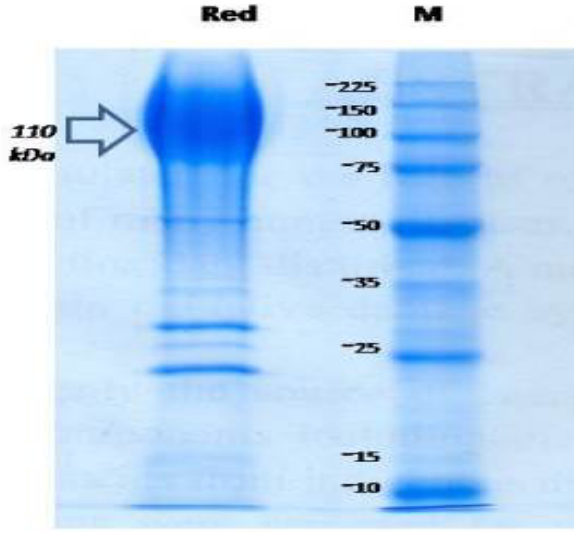

Fig. 12. Purity and molecular mass determination of invertase from *P. expansum* by subjecting to 10% SDS-PAGE. Red: under reduced dissociating/denaturing conditions. Lane M: molecular weight marker (Novagen, USA).

Conclusion

Optimal conditions for the production of invertase (7.03 U/mL) were observed when the strain was grown on culture medium CM1 containing yeast extract as a source of nitrogen, date syrup as a source of carbon after 48 h of incubation at initial pH 5.0, temperature 35º C, inoculum size of $6x10^6$ conidia in 50 mL of culture medium and agitation rate of 150 rev/min.

Invertase from *Penicillium expansum* was also purified and characterized. It was purified to about 6.7 folds than crude enzyme with the recovery of 25.5% having specific activity 215.1 U/mg. The purified invertase was found to have K_m 2.57 mM, V_{max} 178.6 U/mL/min and molecular mass 110 kDa. It has pH and temperature optima 5.5 and 60^{o}C respectively.

Penicillium expansum produced the highest level of invertase and also proved pH (up to 9) and thermo stable (up to 60°C) therefore can be used in industries for invertase production.

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