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Research Article

STUDIES ON ANTI-CANCER ACTIVITY OF LYSYL OXIDASE FROM *TRICHODERMA VIRIDE* MTCC 167

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Abstract

Lysyl oxidase produces H₂O₂ i.e. ROS by acting on L-Lysine. In the present study, ROS thus produced has been used by *in vitro* cytotoxicity assay on ovarian cancer cells thereby achieving 250 percent inhibition. Lysyl oxidase activity was determined spectrophotometrically by Dintrophenylhydrazine (DNPH) reagent. For isolation of lysyl oxidase produced by *Trichodermaviride*, acetone precipitation and ammonium sulphate precipitation was carried out. The effect of temperature on lysyl oxidase production was determined by incubating the media with 1.5 % inoculum at different temperature ranging from 10, 20, 30, 40, 50, 60, 70, 80 °C for 72 hr. Anti-tumor properties of Lysyl oxidase was checked using Rhodamine assay and NBT Assay. Comparative results for Acetone and Ammonium Sulphate precipitation showed that Enzyme activity (U/ml) of acetone precipitation is 124.6 and 144.3 IU/ml and Protein Content is 1.8 and 2.2 mg/ml with the specific activity 68.4 IU/mg and 64.1 IU/mg. The optimum enzyme production was found to be at pH 8 and optimum temperature for lysyl oxidase production was 50°C with maximum enzyme activity of 0.38 (U/ml). 7th fraction contained highest enzyme activity so the retention time of lysyl oxidase was found to be 7 min. The protein content was also calculated by using Bradford method and it was highest in the 1st fraction and in 6th, 7th and 8th fractions. The specific activity has been improved from 75.1 IU/mg to 86.5 IU/mg. 10 units of lysyl oxidase inhibits 3x10⁴ cells in each well to 82.5% and inhibition achieved at same cell count at 200 units which was 250% as observed with NBT and Rhodamine assay.

Keywords: lysyl oxidase; cytotoxicity assay; protein content

Introduction

Lysyl oxidase (LOX) is an extracellular copper dependent enzyme catalyzing lysine-derived cross-linkin extracellular matrix proteins. Protein-lysine 6-oxidase (lysyl oxidase; EC 1.4.3.13) is a copper- containing quinoprotein with lysyl adduct of tyrosylquinone at its active center (Wang et al., 1996). Copper in lysyl oxidase appears to be involved in the transfer of electrons to and from oxygen to facilitate the oxidative deamination of targeted peptidyl lysyl groups in tropocollagen or tropoelastin and to internally catalyse quinone cofactor formation. Oxidation of peptidyl lysine results in the formation of peptidyl α -amino adipic- δ - semialdehyde (Smith et al., 2013). Lysyl oxidase is expressed in different tissues cell types, including fibroblast, aortic and lung smooth muscle cells, osteoblasts, osteosarcoma cells, myofibroblasts (Peyrol et al., 1997), corneal endothelial cells (Fuji et al., 1999), chondrocytes (Greogory et al., 1999) osteosarcoma cells (Uzel et al., 2000). Moreover, a secreted form of lysyl oxidase is associated with tracheal chondrocytes, endothelial cells, basal cells, liver

parenchymal cells, and spleen reticulum cells. Lox also detected in Kidney glomerula, medulla, renal cell lines, and tubular epithelial cells (Donato et al., 1997). At the tissue level LOX is abundantly expressed and immunologically detected in fetal and adult aorta (Baccarani et al., 1989), human placenta, skin and lungs. During the development of different organisms, LOX expression patterns associated with the assembly of collagen and elastin fibers in different tissues, including skin and lung. Studies on developing embryos reported the impact of LOX family members during gastrulation and morphogenesis in various species, including sea urchin, chick, rat and human embryos (Butler et al., 1987) (Casey et al., 1997). LOX has been reported not only to be involved in the cross-linking of collagens and elastin, but to also act as tumor suppressor in transformed fibroblasts and to have intracellular and intranuclear activities (Csizar et al., 2002). Recent evidences suggest that in breast epithelial cells, LOX promotes tumor invasion (Payne et al., 2006). Lysyl oxidase produces H₂O₂ i.e. ROS by acting on L-Lysine. ROS acts as anti-tumor agent. In the present

study, ROS thus produced has been used by *in vitro* cytotoxicity assay on ovarian cancer cells thereby achieving percent inhibition.

Material and Methods

Materials

The microorganisms *Trichoderma viride* was procured from Institute of Microbial Technology, Chandigarh. All the other chemicals and reagents were of analytical grade and were procured from known suppliers of Glaxo India Limited, Hi-media, SD Fine Chemicals, SRL and Ranbaxy.

Methods

Optimization of various parameters for lysyl oxidase production

For maximum production of lysyloxidase from *Trichoderma viride* MTCC 167 various physical and chemical parameters were optimized.

Selection and procurement of microorganisms

Trichoderma viride was grown on specified medium (composition of media as specified in Table 1). The media was sterilized by autoclaving for 15 minutes at 15 psi/cm². The microorganism was maintained on agar slants and was kept in refrigerator at 4.0 ± 0.5 °C for further use. It was sub-cultured after every 7 days. The viability check of microorganism was done after every week by trypan blue. The gram staining was also used to check the contamination in the culture.

Table 1: Composition of growth medium

S.N.	Component	Concentration
1	Malt extract powder	20 g/l
2	Distilled water	1000ml

Optimization of various parameters for lysyl oxidase production

Optimization of pH of the production medium

Five flask containing 25 ml production medium were inoculated with 1.5% inoculum and were maintained at different pH ranging from 2, 3, 4, 5, 6, 7, 8, 9. The flasks were kept at 42 °C in an incubator and optimum pH for lysyl oxidase production from cells of *Trichoderma viride* was determined after 72 hours by evaluating enzyme activity for each pH.

Effect of temperature

The effect of temperature on lysyl oxidase production was determined by incubating the media with 1.5 % inoculum at different temperature ranging from 10, 20, 30, 40, 50, 60, 70, 80°C for 72 days. The optimum temperature was determined by evaluating enzyme activity of all temperature ranges.

Isolation of lysyl oxidase

For isolation of lysyl oxidase produced by *Trichoderma viride*, acetone precipitation and ammonium sulphate precipitation was carried out.

Acetone precipitation: The precipitated protein was centrifuged at 10,000 rpm for 15 minutes at 4°C. 1 ml of ice-cold acetone maintained in water bath was added to 200µl of sample solution, vortexed and incubated at -20°C for 10 minutes. Centrifugation was done for 5 minutes in a microcentrifuge. Supernatant was removed and the pellet was air-dried.

Ammonium Sulphate Precipitation: Placed the beaker of protein solution in a cooling bath on the top of a magnetic

stirrer. While agitating gently on a magnetic stirrer, slowly added 56.8 gm of ammonium sulphate (for 85% protein recovery, 55% by weight ammonium sulphate was added). Continue stirring for 10-30 minutes after salt was added and spinned at 10,000 x g for 10 minutes or at 3000 x g for 30 minutes. Decanted the supernatant and resuspended the precipitates in minimal volumes of buffer. Any insoluble material remaining was probably denatured protein and was removed by centrifugation [13].

Determination of protein concentration

The protein in a sample was determined by using the Bradford method of protein estimation by following procedure of (Bangalore Genei kit). This method is quite sensitive and can detect concentration as low as 20 µg/ml of proteins.

Different volumes of BSA (0.1gm/100ml distilled water) ranging from 100-1000µl were taken in 10 test tubes having 200-2000 µg. The final volume 2000µl was made by adding distilled water. 3ml of Bradford's reagent were added in each test tube and incubated the test tubes at room temperature for 10 minutes. Absorbance was noted at 660nm [14].

Determination of H₂O₂ Concentration by Spectrofluorimetry

Insert the test tube containing the (standard) solution of known concentration into sample holder. Depress the shutter and adjust the standard controls to set the meter to full scale exactly. Note this reading as S. Replace the standard solution with the blank solution. Depress the shutter and noted the meter reading as B. Replace the blank solution with unknown solution. Depress the shutter and noted the reading as U. The blank reading B, is part of reading S and U and must be subtracted before S and U are compared. After subtraction the remainder is proportional to the concentration of considered fluorophor in the standard and the unknown [15].

Concentration of unknown = $(U - B) / (S - B) \times$ Concentration of standard

Determination of enzyme activity

Lysyl oxidase activity can be defined as the µM of lysyl oxidase formed per ml enzyme solution in one minute. Dintrophenylhydrazine (DNPH) reagent determined Lysyl oxidase activity spectrophotometrically. Stock solution of L-Lysine (100mM) was prepared by dissolving 1.8 g of L-Lysine in 100 ml of distilled water.

Purification of enzyme by HPLC

A single injection of standard using 0.45µm filter was made and 30µl of sample was injected with injector valve. A peak for standard was obtained. The chromatograph was operated in a gradient mode, using mobile phase. Peak area and retention time was noted. The retention time was calculated from enzyme activity assay.

SDSPAGE

SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) was performed to determine the molecular weight and the purity of the sample. SDS was performed in 10% to check the molecular weight and purity of enzyme. The protein staining was done by using Commassive brilliant blue [17]. For sample buffer and electrode buffer, composition is mentioned in Table 2 and 3 respectively, other solutions include:

Separating (4x) gel buffer: Tris-HCl (18.3g) was dissolved in 100 ml of distilled water and pH was adjusted 8.8 with IM HCl (Table 4).

Stacking (4x) gel buffer: Tris-HCl (6.055g) was dissolved in 100 ml of distilled water and pH was adjusted 6.8 with IM HCl (Table 5).

Bisacrylamide (30%): 29.2 g acrylamide was mixed with 0.8g of bis-acrylamide and mixture was dissolved in total 100 ml of distilled water.

Sample buffer

Table 2: Composition of Sample buffer

Component	Quantity
Tris-HCl (pH 6.8) buffer	0.4 ml
SDS (10%)	2.5%
2-mercaptoethanol	0.4ml
Glycerol	2.0 ml
Bromophenol blue	0.002 gm
Distilled water	4.7ml

Electrode buffer

Table 3: Composition of Electrode buffer

Component	Quantity
Tris-HCl	6.05 g
SDS	2 gm
Glycine	28.8 gm
Distilled water	2.0 L

Separating gel

Table 4: Composition of separating gel

Component	Quantity
Distilled water	19.5 ml
Bisacrylamide (30%)	10 ml
4x separating gel buffer	10 ml
SDS (10%)	0.8 ml
Glycerol (10%)	0.35 ml
TEMED	20 µl
APS (2%)	0.6 ml

Immediately the whole mixture was poured in a vertical mould and saturated butanol was added and the gel was allowed to polymerize. After ½ hr butanol was removed and upper portion of gel was washed with deionized water.

Stacking gel

Table 5: Composition of stacking gel

Component	Quantity
Distilled water	6.3 ml
Bisacrylamide (30%)	2 ml
4x separating gel buffer	2.5 ml
SDS (10%)	0.2 ml
Glycerol (10%)	0.15 ml
TEMED	10 µl
APS (2%)	0.13 ml

This mixture was poured in vertical moulds of plates on the separating gel. Comb was placed in it and gel was

allowed to settle for 30 minutes. After the stacking gel was polymerized the comb was removed. Sample was prepared by heating in boiling water bath for 2-3 minutes and the sample was loaded in sample wells with the help of auto pipette. Electrophoresis was carried out at 50V up till dye front reached into the separating gel and the voltage was increased to 100V. After the run is complete the gel was taken out and washed with water. Then comassive blue staining was carried out.

The staining solution consisted of 90ml water, 90ml methanol, 10ml acetic acid and 0.25g comassive blue dye. While the destaining solution consisted of 90ml water, 90ml methanol, and 10ml acetic acid

Procedure

Placed the gel in 100 ml of staining solution for 30 minutes for staining the protein in the gel.

Then placed the gel in the destaining solution for destaining the gel for overnight.

Determination of anti-tumor properties of Lysyl oxidase using various cytotoxicity assays

Anti-tumor properties of Lysyl oxidase can be checked with the help of Rhodamine assay (Table 6) and NBT Assay (Table 7).

Rhodamine Assay: The tissue containing cancerous cell (previously washed in buffer) was disaggregated using mechanical methods. The disaggregation was performed in media containing RPMI 1640 and sodium bicarbonate which lead to formation of suspension containing large amount of cells. Then the suspension was added to culture flasks containing media, hormones and antibiotics. Incubated for 24 hours and performed sub culturing of the media having sufficient growth. The sub culturing involves culture flasks containing fresh media and addition of inoculum to them in a sufficient amount. This lead to formation of cell lines from sub culturing of primary culture.

Table 6: Rhodamine assay for lysyl oxidase

	1	2	3	4	5	6	7	8	9	10	11	12
A	C	B	10	10	10	90	90	90	170	170	170	E
B	C	B	20	20	20	100	100	100	180	180	180	E
C	C	B	30	30	30	110	110	110	190	190	190	E
D	C	B	40	40	40	120	120	120	200	200	200	E
E	C	B	50	50	50	130	130	130	E	E	E	E
F	C	B	60	60	60	140	140	140	E	E	E	E
G	C	B	70	70	70	150	150	150	E	E	E	E
H	C	B	80	80	80	160	160	160	E	E	E	E

NBT assay: The tissue containing cancerous cell (previously washed in buffer) was disaggregated using

mechanical methods. The disaggregation was performed in media containing RPMI 1640 and sodium bicarbonate which lead to formation of suspension containing large amount of cells. Then the suspension was added to culture flasks containing media, hormones and antibiotics. Incubated for 24 hours and performed sub culturing of the media having sufficient growth. The sub culturing involves culture flasks containing fresh media and addition of inoculum to them in a sufficient amount. This lead to formation of cell lines from sub culturing of primary culture. The cell lines were counted on haemocytometer. For each experiment the cell count was fixed to 3×10^4 cells which were added to each well.

Table 7: NBT assay for lysyl oxidase

	1	2	3	4	5	6	7	8	9	10	11	12
A	C	B	10	10	10	90	90	90	17	17	17	E
B	C	B	20	20	20	10	10	10	18	18	18	E
C	C	B	30	30	30	11	11	11	19	19	19	E
D	C	B	40	40	40	12	12	12	20	20	20	E
E	C	B	50	50	50	13	13	13	E	E	E	E
F	C	B	60	60	60	14	14	14	E	E	E	E
G	C	B	70	70	70	15	15	15	E	E	E	E
H	C	B	80	80	80	16	16	16	E	E	E	E

For calculating % inhibition following calculations were done:

$$\text{Test O.D} = \text{Test mean} - (\text{B} - \text{E})$$

$$\text{Test mean} = \text{addition of all triplets OD} / 3$$

$$\text{Blank OD (B)} = \text{OD of each (eight) well} / 8$$

$$\text{Control OD (C)} = \text{OD of each (eight) well} / 8$$

$$\text{Empty OD (E)} = \text{OD of each (eight) well} / 8$$

$$\% \text{ inhibition} = \frac{\text{Test OD} - \text{Control OD}}{\text{Control OD}} \times 100$$

Results

Optimization of process parameters

Lysyl oxidase was produced from *Trichoderma viride* and the enzyme activity assay was carried by treating with DNPH. The protein content was calculated by Bradford method. It can be clearly seen from table 8 and fig 1 that as the concentration of keto acid is increased the absorbance also increased. Thus, Absorbance is directly proportional to the concentration. Similar is the case with H₂O₂ (Fig. 2).

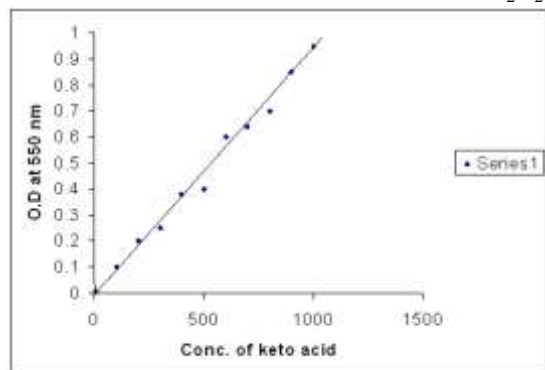


Fig 1: Standard curve for keto acids (100-1000 ppm)

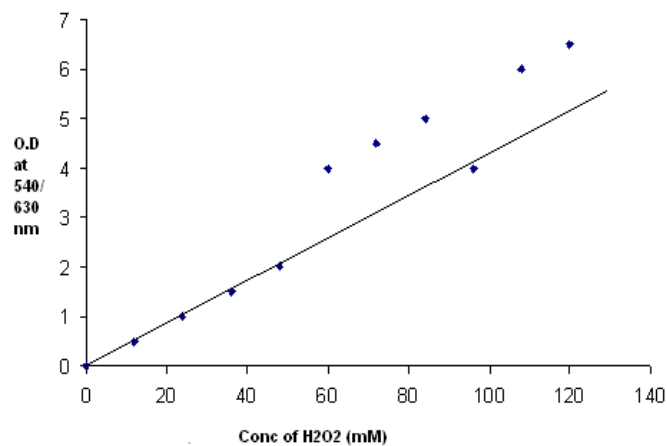


Fig 2: Standard curve for different concentrations of H₂O₂ (12-120 mM)

Standard curve for protein (BSA)

The absorbance for different concentrations of protein (BSA) is given in Table 10. It was found that the absorbance was directly proportional to the BSA concentration, i.e. with increase in concentration of BSA, the absorbance also increased (Fig 3).

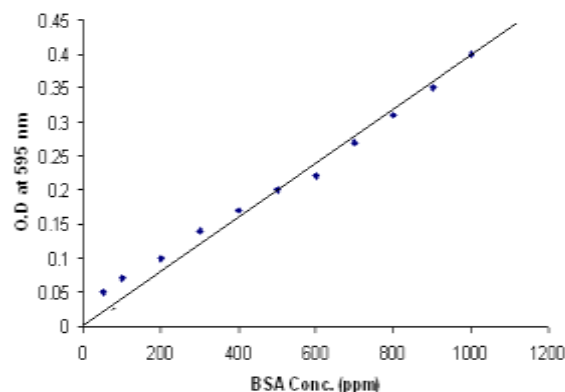


Fig 3: Standard curve for different concentration of protein (BSA).

Isolation of enzyme

By comparing the specific activities of lysyl oxidase from both the methods of purification, it was found that ammonium sulphate precipitation proved to be better than acetone precipitation method because of better enzyme activity and protein content (Table 8).

Table 8: Comparative results for acetone and ammonium sulphate precipitation

S.N.	Sample	Enzyme activity (U/ml)	Protein content (mg/ml)	Specific activity (IU/mg)
1.	Culture (acetone precipitation)	124.6	1.8	68.4
2.	Culture(ammonium sulphate precipitation)	1443.12	2.2	64.1

Optimization of pH for lysyl oxidase production

To observe the effect of pH on lysyl oxidase production the experiment was carried at pH levels (2, 4, 5, 6, 7, 8, 9). From the results, it was observed that optimum enzyme production was found to be pH 8 (Table 9).

Table 9: Effect of pH on lysyl oxidase production from *Trichodermaviride*

S.N.	pH of Medium	O.D. at 540 nm	Enzyme Activity(U/ml)
1.	2	0.2	0.06
2.	3	0.29	0.09
3.	4	0.42	0.21
4.	5	0.57	0.27
5.	6	0.7	0.34
6.	7	0.8	0.40
7.	8	0.95	0.45
8.	9	0.8	0.38
9.	10	0.67	0.32

Effect of temperature on lysyl oxidase production

The effect of temperature on lysyl oxidase was determined by incubating the culture broth at different ranges i.e.10, 20,30,40,50,60,70,80. From the results, it was observed that optimum temperature (Table 10) for lysyl oxidase production was 50°C with maximum enzyme activity of 0.38 (U/ml)

Table 10: Effect of temperature on lysyl oxidase production from *Trichodermaviride*

SNo	Temperature (°C)	O.D. at 540 nm	Enzyme Activity (U/ml)
1.	10	0.3	0.14
2.	20	0.35	0.16
3.	30	0.45	0.21
4.	40	0.6	0.28
5.	50	0.8	0.38
6.	60	0.68	0.33
7.	70	0.52	0.25
8.	80	0.50	0.22

Purification of enzyme by HPLC chromatography

The enzyme activity was calculated in each fraction from 1 to 14 and was found to be higher in the fraction 6th, 7th and 8th (Table 11). 7th fraction contained highest enzyme activity so the retention time of lysyl oxidase was found to be 7 minutes (Fig 4). The protein content was also calculated by using Bradford method and it was highest in the 1st fraction and in 6th, 7th and 8th fractions. The specific activity has been improved from 75.1 IU/mg to 86.5 IU/mg.

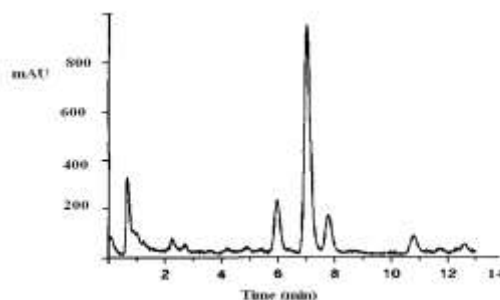


Fig 4: HPLC graph for purification of enzyme

Table 11: Comparison of purified and crude enzyme fractions

Enzyme activity (IU/mg)		Protein Content (mg/ml)	Specific activity (IU/mg)
Crude enzyme	144 units	1.9	75.7
HPLC			
6 th fraction	4	0.43	9.3
7 th fraction	14	0.16	86.5
8 th fraction	6	0.65	9.3

SDS – PAGE

When the SDS-coated proteins travel through the gel during SDS-PAGE, their rate of movement toward the anode is determined almost entirely by size. Because there is a linear relationship between the log of the molecular weight of each protein and its migration distance, SDS-PAGE was used to estimate the molecular weight of each protein based on its migration distance. It can be clearly seen from Fig 5 that lane 2,3 and 4 are 6th, 7th and 8th fractions respectively while lane 1 is the marker for HPLC fraction and it can be observed that the molecular weight of the lysyl oxidase is between 25-100KDa.

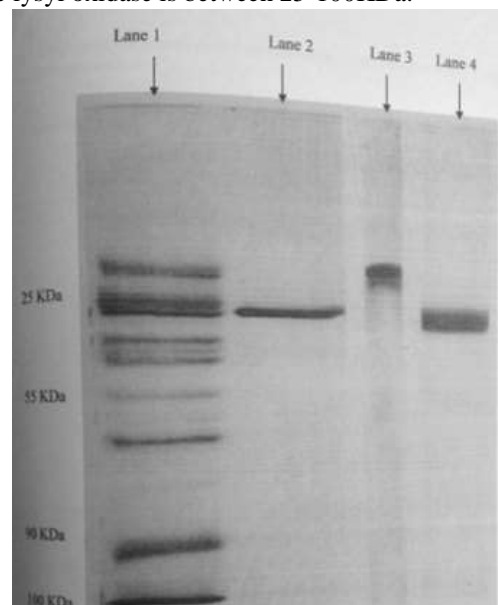


Fig 5: SDS Page

Determination of anti-tumor properties of Lysyl oxidase

10 units of lysyl oxidase inhibits 3×10^4 cells in each well to 82.5% and inhibition achieved at same cell count at 200 units which was 250%. When the units were increased further, the same inhibition was achieved (Fig 6 and 7).

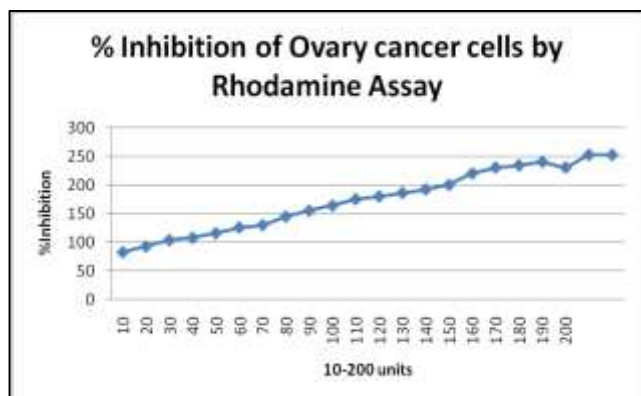


Fig 6: Percent inhibition of ovary cancer cells by rhodamine assay

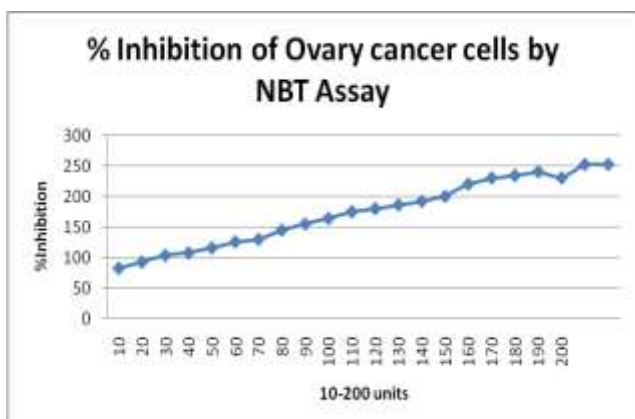


Fig 7: Percent inhibition of ovary cancer cells by rhodamine assay

Summary

Lysyl oxidase (LOX) is an extracellular copper dependent enzyme catalyzing lysine-derived cross-links in extracellular matrix proteins. Lysyl oxidase was found to be isolated from a number of sources such as animal tissues and microorganisms. Lysyl oxidase is an important enzyme that serves many functions in our daily life. It was found to play a key role in tumor suppression. It acts as tumor suppressor in transformed fibroblasts and to have intracellular and intranuclear activities. Recent evidences suggest that in breast epithelial cells, LOX promotes tumor invasion. LOX is a potent tumor suppressor gene in fibroblasts, basal, and squamous cell and gastric carcinomas. It was found evenly distributed throughout the stroma of the chroid plexus and was also detected in the ependymal and subependymal layers of the ventricle walls. The formation of collagen or elastin cross-links by LOX leads to an increase in tensile strength and structural integrity which is essential for normal connective tissue function. Lysyl oxidase plays a pivotal role in embryogenesis and development. A lysyl oxidase enzyme from *Trichoderma viride* was isolated. The Lysyl oxidase production was found to be enhanced by increasing the level of lysine. The parameters were optimized such as pH, temperature for maximum production of lysyl oxidase. Under these optimum conditions, by acetone precipitation and ammonium sulphate precipitation, the enzyme was isolated and was purified by High performance liquid chromatography. In

Vitro Cytotoxicity assays (NBT and Rhodamine) on ovarian cancer cells was performed and % Inhibition was achieved.

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