ANNEXURE I:

Protocols & Definitions

1.1 PREPARATION OF BUFFERS SOLUTIONS

1. ACETATE BUFFER

Stock solutions

A: 0.2 M solution of acetic acid (11.55 ml in 1000 ml)

B: 0.2 M solution of sodium acetate (16.4 gm of C_2H_2Na or 27.2 gm of $C_2H_3O_2Na.3H_2O$ in 1000 ml).

x ml of A, y ml of B, diluted to a total of 100 ml.

x	y	pH
46.3	3.7	3.6
44.0	6.0	3.8
41.0	9.0	4.0
36.8	13.2	4.2
30.5	19.5	4.4
25.5	24.5	4.6
20.0	30.0	4.8
14.8	35.2	5.0
10.5	39.5	5.2
8.8	41.2	5.4
4.8	45.2	5.6

2. BORIC ACID-BORAX BUFFER

Stock solutions

A: 0.2 M solution of boric acid (12.4 gm in 1000 ml)

B: 0.05 M solution of borax (19.05 gm in 1000 ml; 0.2 M in terms of sodium borate).

50 ml of A, x ml of B, diluted to a total of 200 ml.

x	pH
2.0	7.6
3.1	7.8
4.9	8.0
7.3	8.2
11.5	8.4
17.5	8.6
22.5	8.7
30.0	8.8
42.5	8.9

59.0	9.0
83.0	9.1
115.0	9.2

3. CARBONATE-BICARBONATE BUFFER

Stock solutions

A: 0.2 M solution of anhydrous sodium carbonate (21.2 gm in 1000 ml)
B: 0.2 M solution of sodium bicarbonate (16.8 gm in 1000 ml).
x ml of A, y ml of B, diluted to a total of 200 ml.

x	y	pH
4.0	46.0	9.2
7.5	42.5	9.3
9.5	40.5	9.4
13.0	37.0	9.5
16.0	34.0	9.6
19.5	30.5	9.7
22.0	28.0	9.8
25.0	25.0	9.9
27.5	22.5	10.0
30.0	20.0	10.1
33.0	17.0	10.2
35.5	14.5	10.3
38.5	11.5	10.4
40.5	9.5	10.5
42.5	7.5	10.6
45.0	5.0	10.7

4. CITRATE BUFFER

Stock solutions

A: 0.1 M solution of citric acid (21.01 gm in 1000 ml)
B: 0.1 M solution of sodium citrate (29.41 gm $C_6H_5O_7Na_3.2H_2O$ in 1000 ml).
x ml of A, y ml of B, diluted to a total of 100 ml.

x	y	pH
46.5	3.5	3.0
43.7	6.3	3.2

40.0	10.0	3.4
37.0	13.0	3.6
35.0	15.0	3.8
33.0	17.0	4.0
31.5	18.5	4.2
28.0	22.0	4.4
25.5	24.5	4.6
23.0	27.0	4.8
20.5	29.5	5.0
18.0	32.0	5.2
16.0	34.0	5.4
13.7	36.3	5.6
11.8	38.2	5.8
9.5	40.5	6.0
7.2	42.8	6.2

5. GLYCINE-HCl BUFFER

Stock solutions
A: 0.2 M glycine (15.01 gm in 1000 ml)
B: 0.2 M HCl
25 ml of A, x ml of B, diluted to a total of 100 ml.

x	pH
22.0	2.2
16.2	2.4
12.1	2.6
8.4	2.8
5.7	3.0
4.1	3.2
3.2	3.4
2.5	3.6

6. PHOSPHATE BUFFER

Stock solutions
A: 0.2 M solution of monobasic sodium phosphate (27.8 gm in 1000 ml)
B: 0.2 M solution of dibasic sodium phosphate (53.65 gm $Na_2HPO_4.7H_2O$ or $Na_2HPO_4.12H_2O$ in 1000 ml).

x ml of A, y ml of B, diluted to a total of 200 ml.

x	y	pH
93.5	6.5	5.7
92.0	8.0	5.8
90.0	10.0	5.9
87.7	12.3	6.0
85.0	15.0	6.1
81.5	18.5	6.2
77.5	22.5	6.3
73.5	26.5	6.4
68.5	31.5	6.5
62.5	37.5	6.6
56.5	43.5	6.7
51.0	49.0	6.8
45.0	55.0	6.9
39.0	61.0	7.0
33.0	67.0	7.1
28.0	72.0	7.2
23.0	77.0	7.3
19.0	81.0	7.4
16.0	84.0	7.5
13.0	87.0	7.6
10.5	89.5	7.7
8.5	91.5	7.8
7.0	93.0	7.9
5.3	94.7	8.0

7. TRIS (HYDROXYMETHYL) AMINOMETHANE (TRIS HCl) BUFFER

Stock solutions
A: 0.2 M solution of Tris (hydroxymethyl) aminomethane (24.2 gm in 1000 ml)
B: 0.2 M HCl
50 ml of A, x ml of B, diluted to a total of 200 ml.

x	pH
5.0	9.0
8.1	8.8

12.2	8.6
16.5	8.4
21.9	8.2
26.8	8.0
32.5	7.8
38.4	7.6
41.4	7.4
44.2	7.2

8. GLYCINE-NaOH BUFFER

Stock solutions
A: 0.2 M solution of glycine (15.01 gm in 1000 ml)
B: 0.2 M NaOH
50 ml of A + x ml of B, diluted to a total of 200 ml.

x	pH
4.0	8.6
6.0	8.8
8.8	9.0
12.0	9.2
16.8	9.4
22.4	9.6
27.2	9.8
32.0	10.0
38.6	10.4
45.5	10.6

CONCENTRATION OF SOLUTION:

The concentration of the solution that is prepared and used in biological experiments can be expressed in several ways. The most commonly used expressions and the solution preparation are given below.

1. **Molality**
   The molality (M) of the solution is defined as the number of moles of substance per liter, thus, having the dimensions of mol/l. It can be expressed as:

$$\text{Molality(M)} = \frac{\text{Number of the moles of solute}}{\text{1 liter of solution}}$$

$$\text{Molality(M)} = \frac{\text{Number of the millimoles of solute}}{\text{1 milliliter of solution}}$$

2. **Per cent Solutions**
   Per cent solutions are frequently used in biological preparation. It can be a source of confusion because per cent solutions can be expressed in many different ways. Thus, it is important to specify the per cent type (e.g., w/v) when dealing with per cent solutions.

   a. Weight per cent (w/w):
   Weight per cent refers to the number of grams of solute per 100 grams of solution. For a 20 % (w/w) solution, dissolve 20 grams of the substance in 80 grams of solvent or water. Mathematically,

   Weight percent (w/w) = mass solute/mass solution x 100 %

   b. Volume per cent (v/v):
   Volume per cent refers to the number of milliliters of a liquid substance in 100 ml of solution. For a 20 % (v/v) solution, dissolve 20 ml of liquid solute in 80 ml of solvent or water. Mathematically,

   Volume percent (v/v) = volume solute/volume solution

   c. Weight per volume (w/v):
   Weight per volume percent is often employed to indicate the composition of dilute aqueous solutions of solids. Also, referred to as the number of grams of a substance in 100 ml of solution. For a 20 % solution, dissolve 20 grams of a substance in water to a final volume of 100 ml. mathematically,

$$\text{Weight/volume (w/v)} = \frac{\text{Mass of solute (g)}}{\text{Volume solution (ml)}} \text{ x } 100\ \%$$

3. Normality

The normality (N) can be defined as the number of gram equivalent weight of a substance per liter of solution (i.e., molecular weight divided by the hydrogen equivalent of the substance). The gram equivalent weight of a substance depends on what the nature of reaction that the solution is to be used for.
The relationship between molality and normality can be mathematically represented as,

$$\text{Normality} = \text{Molality} \times \text{Valence}$$

[Valence is the charge of hydrogen or positive ions, in respect to the negative ions of a compound.]

4. Dilutions

To make dilutions of a solution, the following equation should be employed:

$$M_i \times V_i = M_f \times V_f$$

[$_i$ = initial; $_f$ = final; M = Molarity; V = Volume]

NOTES:

pH: pH is a value taken to represent the acidity or alkalinity of an aqueous solution. It is defined as logarithm of the reciprocal of the hydrogen ion concentration of the solution

$$\text{i.e., } pH = \log \frac{1}{[H^+]}$$

Atomic weight: The atomic weight of an element is the relative weight of the atom on the basis of oxygen as 16.

Molecular weight: The sum of the atomic weights of all the atoms in a molecule is its molecular weight.

Equivalent weight: The equivalent weight of a substance is the number of grams of the substance required to react with, replace or furnish one mole of H_2O^+ or OH^-.
The equivalent weight of an acid is the wight that contains one atomic weight of acidic hydrogen i.e., the hydrogen that reacts during neutralization of acid with base.

Units of Length:
1 micron = 1 μ = 1 μm = 1 x10^{-6} m = 1 x 10^{-3} nm = 1 x 10^4 Å
1 Å = 0.1 nm = 1 x 10^{-4} μm = 1 x 10^{-10} m
1 nm = 10 Å = 1 x 10^{-3} μm = 1 x 10^{-9} m

1.2. PREPARATION OF MEDIA

Sl. No.	Media	Composition and Preparation
A	**NATURAL MEDIA**	
1	Waterhyacinth leaf extract (WhDA)*	Waterhyacinth leaves 150 g Distill water 1000 ml Agar 20 g (for solid media) Dextrose 8 g (Crush and boil 150g of the leaves in distilled water. Filter and raise the volume to 1000ml and then add 8 g dextrose and agar)
B	**SEMI-SYNTHETIC MEDIA**	
2	Potato Dextrose media	Potato 200 g Dextrose 20 g Agar 20 g (for solid media) Distilled water 1000 ml Slice and boil the potatoes for an hour in distilled water. Filter it using muslin cloth. Add dextrose and agar (for solid media). Autoclave for 15 min at 121 ^{o}C
3	Martiin's media	Dextrose 20 g Peptone 5 g KH_2PO_4 1 g $MgSO_4.7H2O$ 0.5 g Agar 17 g (for solid media) Distilled water 1000 ml
4.	Sabouraud's Media	Dextrose 40 g Peptone 10 g Agar 17 g (for solid media) Distilled water 1000 ml
5.	Maltose Peptone	Maltose 1.6 g Peptone 55.6 g Agar 15 g (for solid media) Distilled water 1000 ml
6	Yeast Glucose	Yeast extract 1 g Glucose 3 g Na_2HPO_4 0.6 g KH_2PO_4 1.4 g $MgSO_4.7H2O$ 0.1 g Agar 17 g Distilled water 1000 ml
7	Malt Extract	Malt Extract 20 g Dextrose 20 g Peptone 1 g

		Agar Distilled water	25 g (for solid media) 1000 ml
C	**SYNTHETIC MEDIA**		
8	Czapek's	$NaNO_3$ K_2HPO_4 $MgSO_4.7H2O$ KCl Sucrose Agar Distilled water	2 g 2.95 g 0.5 g 0.5 g 30 g 15 g (for solid media) 1000 ml
9	Miller's	Dextrose Peptone Yeast extract Agar Distilled water	0.5 g 0.5 g 30 g 15 g 1000 ml
10	Mayer's	$MgSO_4$ NH_4NO_3 $CaPO_4$ KH_2PO_4 Sucrose Agar Distilled water	2.5 g 10 g 2.5 g 5 g 50 g 17 g (for solid media) 1000 ml
11	Lenolin	Peptone Maltose Malt extract KH_2PO_4 $MgSO_4.7H2O$ Agar Distilled water	0.0625 g 6.25 g 6.25 g 1.25 g 0.625 g 17 g (for solid media) 1000 ml
12	Richard's	Sucrose KNO3 KH_2PO_4 $MgSO_4.7H2O$ $FeCl_3$ Agar Distilled water	35 g 10 g 5 g 2.5 g trace 17 g (for solid media) 1000 ml
13	Asthana & Hawker	Dextrose KNO_3 KH_2PO_4 $MgSO_4.7H2O$ Agar Distilled water	0.5 g 3.5 g 1.75 g 0.75 g 17 g (for solid media) 1000 ml
14.	Cohn's	KH_2PO4 $MgSO_4.7H2O$ Ammonium tartrate KCl Agar Distilled water	5 g 0.5 g 10 g 0.5 g 17 g (for solid media) 1000 ml
15	Nutrient Media	Peptone Yeast/beef extract	5 g 3 g

		NaCl Agar Distilled water	5 g 15 g 1000 ml
		pH is adjusted to neutral (7) at 25 ^{o}C	
16	Rose Bengal Chloramphenicol Media	Glucose Mycologic polypeptone KH_2PO_4 Chloramphenicol Rose Bengal Dye Agar Distilled water	10 g 5 g 1 g 0.2 g 0.06 g 17 g (for solid media) 1000 ml

The Basal Media is composed of

Calcium Chloride	0.2 g
Magnesium Sulphate (anhydrous)	0.09767 g
Potassium Chloride	0.4g
Sodium Bicarbonate	2.2 g
Sodium Chloride	6.8 g
Sodium Phosphate Monobasic (anhydrous)	0.122
Agar	17 g (for solid media)
Distilled water	1000 ml

*[The WhDA was prepared as follows: fresh-water hyacinth leaves were washed in running tap water and then in distilled water. They were chopped into small fragments and boiled for 20–25 min in 500 ml distilled water and filtered through cheesecloth for the collection of extract. Dextrose and agar-agar were added to this extract and boiled until transparent.]

1.3 PROCEDURE FOR STANDARD CURVE AND QUANTITATIVE ENZYME ESTIMATION BY DNS METHOD

INTRODUCTION

Sugars with reducing property (arising out of the presence of a potential aldehyde or keto group) are called reducing sugars. Some of the reducing sugars are glucose, galactose, lactose and maltose. Dinitrosalicylic acid method is a simple, sensitive and adoptable method for handling large number of samples at a time.

MATERIALS AND REAGENTS REQUIRED

1. Test Tubes
2. Pipettes
3. Water Bath
4. Colorimeter
5. 3,5- Dinitrosalisylic Acid
6. Glucose/Maltose Working Solution
7. 2 (N) NaOH
8. Distill water

PREPARATION OF REAGENTS

1. 3,5 dinitrosalisylic acid (DNS): About 1 gm of DNS is dissolved in 50 ml of distilled water. To this solution add 30 gm of Sodium Potassium Tartarate tetrahydrate in small lots, the solution turns milky yellow in colour. Then add 20 ml of 2 (N) NaOH, which turns the solution to transparent orange-yellow colour. The final volume is made to 100 ml with the distilled water. This solution is stored in an amber-coloured bottle.
   [DNS-Dinitrosalisylic Acid Preparation
   50 ml water+15 gm Na-K Tartarate + 0.8 gm of NaOH pellets + 0.5 gm of DNSA
   ⟶ Mix with magnetic stirrer.]
2. Maltose/Glucose Working Solution: 180 mg of maltose is weighed and made up to 100 ml with distilled water.

PROCEDURE

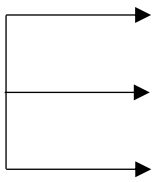
a. For standard curve

1. Pipette out standard maltose solution in the range of 0.2, 0.4, 0.6, 0.8 and 1 ml, in 5 separate test tubes.
2. A test tube containing a blank solution is also prepared.
3. Using distilled water, bring the volume up to 2 ml in each test tube, including the test tube containing the blank solution.
4. Add 1 ml of DNS reagent to each tube and cover the test tubes with aluminum foil.
5. Heat the contents in the test tube in a boiling water bath for 5 minutes.
6. Cool the test tubes to room temperature, after taking them out of the water bath.
7. Then add 9 ml distilled water to each test tube and mix well.

8. Take 1 ml from each test tube into different cuvettes and place each cuvette in a colorimeter and record the intensity of dark orange red colour at 540 nm as the absorbance optical density (OD).
9. Plot a graph with the amount of maltose on X-axis v/s the OD at 540 nm on Y-axis.

b. For quantitative enzyme estimation

1. Filter the flasks/test tubes, incubated, individually with Whatmann's Filter Paper
2. The collected filtrate is the respective enzymes produced.
3. Take the enzyme/soup (1.5 ml soup) into the respective appendorf, and check the pH.
4. Centrifuge the enzymes; discard the pellet (let it remain in the appendorf itself)
5. Prepare 3 test tubes for each appendorf/enzyme to check their activity.
6. In the meantime, prepare substrate solution (0.1 gm/100 ml or 1 gm/100 ml or 1 % w/v- as according to the substrate concentration given for enzyme production by the fungus) for all individuals substrates.
7. 3 test tubes



(+) ve = 250 µl enzyme + 250 µl substrate

(-) ve = 250 µl enzyme + 250 µl water

blank = 250 µl substrate + 250 µl water

8. Incubate all the test tubes for 37 ^{o}C, for 10 minutes.
9. Mix 500 µl DNSA for each test tubes and boil for 8-12 minutes and then add 5 ml water individually
10. Measure the optical density at 540 nm, at colorimeter.

RESULT

1. The intensity of the colour observed will be proportional to the concentration of maltose present in the solution.
2. OD value measured at colorimeter can be converted to enzyme unit from the R^2 value obtained from the standard curve prepared.

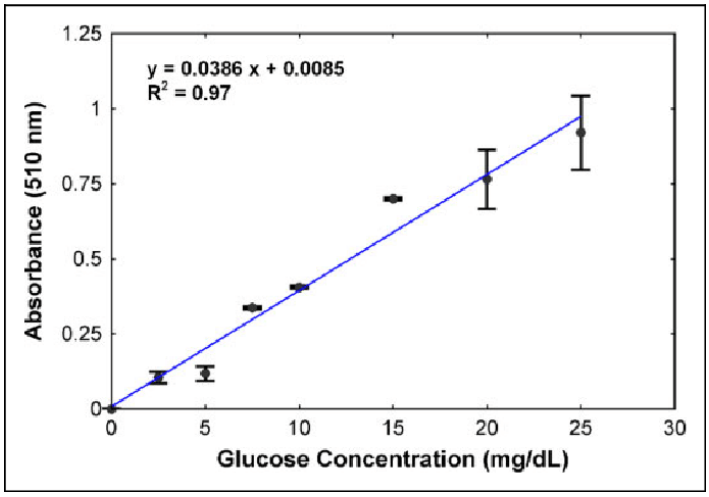


Figure: Sample standard curve for glucose concentration

1.4. AMMONIUM SULPHATE FRACTIONATION OF PROTEINS

INTRODUCTION

The solubility of proteins is markedly affected by the ionic strength of the medium. As the ionic strength is increased, protein solubility at first increases. This is referred as 'salting in'. However, beyond a certain point the solubility begins to decrease and this is known as 'salting out'.

At low ionic strengths the activity coefficients of the ionizable groups of the proteins are decreased so that their effective concentration is decreased. This is because the ionizable groups become surrounded by counter ions which prevent interaction between the ionizable groups. Thus protein-protein interactions are decreased and the solubility is increased.

At high ionic strengths much water becomes bound by the added ions that not enough remains to properly hydrate the proteins. As a result, protein-protein interactions exceed protein-water interactions and the solubility decreases.

Because of differences in structure and amino acid sequence, proteins differ in their salting in and salting out behavior. This forms the basis for the fractional precipitation of proteins by means of salt.

Ammonium sulphate is a particularly useful salt for the fractional precipitation of proteins. It is available in highly purified form, has great solubility allowing for significant changes in the ionic strength and is inexpensive. Changes in the ammonium sulphate concentration of a solution can be brought about either by adding solid substance or by adding a solution of known saturation, generally, a fully saturated (100 %) solution.

Table: Quantities of ammonium sulphate required to reach given degrees of saturation at +20 °C.

	Final percent saturation to be obtained																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
Starting percent saturation	Amount of ammonium sulphate to add (grams) per liter of solution at +20 °C																
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	85	115	146	179	212	246	282	319	358	397	439	481	526	572	621	671	723
10	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
15	28	58	88	119	151	185	219	255	293	331	371	413	456	501	548	596	647
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
25		0	29	60	91	123	157	191	228	265	304	344	386	429	475	522	571
30			0	30	61	92	125	160	195	232	270	309	351	393	438	485	533
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495
40					0	31	63	96	130	166	202	241	281	322	365	410	457
45						0	31	64	98	132	169	206	245	286	329	373	419
50							0	32	65	99	135	172	210	250	292	335	381
55								0	33	66	101	138	175	215	256	298	343
60									0	33	67	103	140	179	219	261	305
65										0	34	69	105	143	183	224	267
70											0	34	70	107	146	186	228
75												0	35	72	110	149	190
80													0	36	73	112	152
85														0	37	75	114
90															0	37	76
95																0	38

MATERIALS REQUIRED

Solutions needed for precipitation:

1. Saturated ammonium sulphate solution (add 100 g ammonium sulphate to 100 ml distilled water, stir to dissolve).
2. 1 M Tris-HCl, pH 8.0.
3. Buffer for first purification step.

PROCEDURE

1. Filter (0.45 µm) or centrifuge the sample (10 000 rpm at +4 °C).
2. Add 1 part 1 M Tris-HCl, pH 8.0 to 10 parts sample volume to maintain pH.
3. Stir gently.
4. Add ammonium sulphate solution, drop by drop. Add up to 50 % saturation*. (Fine crushed ammonium sulphate could be also added slowly and steadily to avoid lump formation or increased local concentration of the salt)
5. Stir for 1 hour or until thoroughly mixed and no trace of crystals appear
6. Centrifuge 25 minutes at 10 000 rpm at +4 °C.
7. Remove supernatant. Wash the pellet twice by resuspension in an equal volume of ammonium sulphate solution of the same concentration (i.e. a solution that will not redissolve the precipitated protein or cause further precipitation).
8. Centrifuge again.
9. Dissolve pellet in a small volume of the buffer to be used for the next step. (Until the next process the pellet mixed with buffer can be refrigerated overnight).
10. Ammonium sulphate is removed during clarification/buffer exchange steps with Sephadex G-25, using desalting columns

*The % saturation can be adjusted either to precipitate a target molecule or to precipitate contaminants.

PRECAUTIONS

1. Some proteins may be damaged by ammonium sulphate. Take care when adding crystalline ammonium sulphate: high local concentrations may cause contamination of the precipitate with unwanted proteins.
2. For routine, reproducible purification, precipitation with ammonium sulphate should be avoided in favor of chromatography.
3. In general, precipitation is rarely effective for protein concentrations below 1 mg/ml.

1.5. GEL-FILTRATION CHROMATOGRAPHY

INTRODUCTION

Gel filtration chromatography (sometimes referred to as size exclusion chromatography) separates biomolecules based on differences in their molecular size. The process employs a gel media suspended in an aqueous buffer solution which is commonly packed into a chromatographic column. These columns can vary in size from very small (for example, spin columns of <1 ml bed volume for analytical separations) to very large (for preparative scale applications). The gel media consists of spherical porous particles of carefully controlled pore size through which biomolecules diffuse to different extents based on differences in their molecular sizes. Small molecules diffuse freely into the pores and their movement through the column is retarded, whereas large molecules are unable to enter the pores and are therefore eluted earlier. Hence, molecules are separated in order of decreasing molecular weight, with the largest molecules eluting from the column first.

Size exclusion column bed has three functional components: the pore volume, the void volume, and the matrix volume (bed volume). The pore volume refers to the pore-lumen space within the particles. The void volume refers to the excluded volume i.e., the space between the particles. And the matrix volume refers to the solid component of the particles that fills the column bed.

The pore diameter defines the exclusion limit of the gel. Proteins too large to enter the pores are excluded and have access to only the void volume. Proteins larger than the exclusion limit elute together in a single peak at the beginning of the chromatography: the void volume. Protein molecules that are smaller than the pore size enter the particles and their separation is determined by the pore size distribution within the pore volume. Among these proteins, the larger proteins are eluted earlier than the smaller protein molecules giving rise to the fractionation of protein molecules based on their size.

The protein molecules to be fractionated must have an opportunity to diffuse in and out of the pores and therefore the flow rate of the sample entering the column is critical. The importance of having a suitable diffusion time makes size exclusion chromatography is the slowest of the fractionation techniques.

MATERIALS REQUIRED

1. Chromatography buffer stock solution:
   a. 1 M Tris,
   b. pH 7.5.
   c. Distill water
   d. 121.1 g Tris (see Note 1).
   e. HCl.

   Note:

   To 1 M Tris of pH 7.5, add about 600 ml water to a glass beaker and dissolve 121.1 gm Tris. When completely dissolved, adjust pH to 7.5 with HCl. Add water to a final volume of 1000 ml. Then store at 4 °C.
2. Salt stock solution:
   a. 5 M NaCl.
   b. Distill water.

Note:
For 5 M NaCl, weigh 292.2 g NaCl and add to a beaker already containing about 500 ml water. Stir to dissolve, and add water to a final volume of 1000 ml Store at room temperature.

3. Chromatography buffer:
    a. 20 mM Tris, pH 7.5,
    b. 100 mM NaCl.
    c. Distill water

    Note:
    20 mM Tris, pH 7.5 is prepared and 100 mM NaCl.
    Combine 20 ml 1 M Tris, pH 7.5 with 20 ml 5 M NaCl and 960 ml water. Store at 4 °C.
4. Gel filtration column:
    a. Sephadex G-25, which is appropriate for separation of molecules of the molecular weight 1,000 to 5,000 range (with an exclusion limit of approximately 5000. Proteins and peptides larger than 5000 are therefore easily separated from molecules with molecular weights of less than 1000)
5. Molecular weight standards (Optional):
    We use a gel filtration standard (Bio-Rad), which is a mixture of bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B-12 (1.4 kDa).

PROCEDURE

All procedures are carried out at room temperature, unless noted otherwise.

1. Clamp the Size Exclusion Column in an upright position to the stand
2. First run distill water for 4-5 times and ensure proper channeling of beads for later buffer and sample loading.
3. Cut the tip and allow water to drain several times.
4. Open the top cap first and then the bottom cap of the column to prevent air entering the resin. Allow the buffer to drain out of the column, under gravity, to a waste container. Ensure that the resin settles evenly in the column.
5. Equilibrate the column: Apply 1000 µl gel filtration chromatography buffer to the top of the column and allow it to drain out freely into a waste container. Repeat this step 9 more times, so that a total of 10 ml Gel Filtration Buffer has been added. (gel filtration buffer here is Acetate Buffer)
6. Run gel filtration standards to calibrate the column. Record the elution volume of the standards
7. Apply protein sample to the column. Carefully load 2.5 ml gel filtration sample (here the partially purified xylanase enzyme) to the column without disturbing the column surface and in capped condition. Allow the sample to enter the column. (The volume of the sample should be less than 5 % of the column volume, preferably with a concentration of 5-10 mg/ml.)
8. Run the 2.5 ml sample through the column (ensuring no pass out from the tip);
9. Elute the sample: Then open the cap once the sample clears the level of bead of column, add 3.5 ml gel filtration buffer to the top of the column, in the capped condition and allow it to drain freely.

10. Elute the protein with a flow rate of 0.5 ml/min. (Record the elution volume of the protein).
11. Repeat the elution step until sample begins to elute from the column. When the brownish xylanase starts to elute from the column, position a centrifuge tube under the column and collect 0.2-0.3 ml elution into the tube. Collect a few fractions until all brownish xylanase has been eluted from the column.
12. This fraction is the protein fraction and can be refrigerated for further uses. Do check the activity of the protein, prior to experimentation
13. Create a standard curve by plotting the log molecular masses of the standards versus Ve/V0, where Ve represents the elution volume, V0 the void volume.

NOTES

1. To facilitate dissolution of Tris, add water first to avoid Tris sticking to the bottom of the beaker and preventing the magnetic stir bar from spinning.
2. Tris is an excellent buffer, and a significant volume of HCl is required to adjust the pH to 7.5. Therefore, be sure not to add too much water to the beaker while solubilizing Tris. Use concentrated HCl. When approaching the desired pH, dilute (1 M) HCl may be used for better control.
3. The solution may need to be heated to dissolve the NaCl. Since the solution is nearly saturated, add water to almost the desired final volume while dissolving.
4. For gel filtration chromatography, Tris buffer or sodium phosphate buffer is most commonly used. An ionic strength of at least 0.05 M is recommended to reduce nonspecific interactions between the proteins being separated and the chromatographic matrix. The exact buffer composition may need to be determined empirically, as it should not cause protein inactivation or precipitation.
5. Several commercially available resins exist that are suitable for different fractionation ranges. The most suitable resin should be selected based on the required separation range.
6. A very large molecule such as thyroglobulin is used to determine the void volume.
7. The sample volume should be less than 5 % of the column volume to ensure elution as a sharp peak; the higher the ratio of sample to column volume, the lower the resolution. The sample buffer need not be the same as the chromatography buffer.
8. The elution volumes of the standards are divided by the elution volume of thyroglobulin (Ve/Vo) and plotted against the log of the molecular weight of the individual standards. The elution volume decreases approximately linearly with the logarithm of the molecular weight.

NB:

Standardization and standard graph preparation were ignored, as gel-filtration chromatography method was used to further purification of the enzyme and remove the ammonium sulphate salts and elute out fractions of proteins for a particular molecular weight, as per literature review of the xylanase enzyme.

1.6. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS

INTRODUCTION

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most commonly used method to fractionate polypeptides on the basis of their molecular sizes in an electrical field. The gel matrix in polyacrylamide gel provides small pore sizes (actual size depends upon the concentration of polyacrylamide and the ratio of acrylamide to bis-acrylamide) which allows a clear separation of polypeptides of varying lengths along the length of a gel. The use of ionic detergent like sodium-do-decyl-sulphate (SDS) helps to eliminate secondary structures of the polypeptides and also provides a net negative charge to each polypeptide so that all of them move towards the anode (positive pole) when a voltage gradient is applied to the gel. Due to these features, the mobility of a polypeptide in SDS-PAGE is directly proportional to its molecular size (polypeptide length). The chart below shows the linear range of mobility of polypeptides of different sizes (in kilodaltons, kDa) in polyacrylamide ration being 29:1 in all cases):

% Acrylamide Concentration	Size (in kDa) in Linear Range Separation
15 %	12 to 43 kDa
10 %	16 to 68 kDa
7.5 %	36 to 94 kDa
5.0 %	57 to 212 kDa

Thus, depending upon the size of polypeptides that are desired to be resolved, a gel with corresponding concentration of acrylamide may be prepared.

After the electrophoresis, the proteins can be conveniently visualized by staining the gel with stains like Coommassie Blue of Silver etc. The electrophoresed proteins may also be transferred to nitrocellulose or nylon membranes for immunological detection of specific proteins/polypeptides (Western Blotting) or if these are labelled with ^{35}S-methionine (or other radioactively labelled amino acids), these may be detected by autoradiography (or fluorography) of the dried gel.

MATERIALS REQUIRED

1. Acrylamide Stock Solution (acrylamide: bis-acrylamide :: 29:1)
    a. Acrylamide 29 gm
    b. N,N'-methylene- bis-acrylamide 1 gm
    c. Deionised water 100 ml
    Warm the warm to assist dissolution of acrylamide. Store the stock solution in dark bottle at 10 ^{o}C.
2. Sodium-dodecyl sulphate (SDS) stock solution
    a. SDS 10 gm
    b. Deionized water to make 100 ml
    Store at room temperature
3. Tris Buffers (1 M stock) -pH 8.8 and 6.8

a. Tris base 12.1 gm
b. Distilled water 100 ml
Adjust to pH 8.8 or to 6.8 with 1 N HCl

4. Ammonium persulphate (APS) stock solution
   a. Ammonium persulphate 10 gm
   b. Distill water to make 100 ml
5. N,N,N',N'-tetramethylene diamine (TEMED)- stock stored at 4 ^{o}C in dark bottle
6. Tris Glycine Electrophoresis Buffer (5X)
   a. Tris Base 15.1 gm
   b. Glycine 94.0 gm
   c. Distill water to make 100 ml
   (let the contents dissolve fully before adding SDS solution and water)
   d. 10 % SDS 50 ml
   e. Distill water to make 100 ml
   pH should be 8.3
7. 3 M Sodium Acetate (pH 5.2)
   a. Sodium acetate 40.81 gm
   b. Distill water 80 ml
   Dissolve and adjust pH to 5.2 with Glacial Acetic Acid and make the volume to 100 ml
8. 10 mM Sodium Acetate (pH 5.2)
   a. 3 M Sodium acetate (pH 5.2) 3.3 µl
   b. Distill water to make 1 ml
9. 1 M Dithiothreitol (DTT)
10. 100 mM Phenyl methyl sulphonyl fluoride (PMSF)
    a. PMSF 17.4 mg
    b. Absolute Ethanol 1 ml
11. Sample Buffer
    a. 1 M Tris (pH 6.8) 50 µl
    b. 1 M DTT 100 µl
    c. 10 % SDS 200 µl
    d. 1 % Bromophenol Blue 100 µl
    e. Glycerol 100 µl
    f. 100 mM PMSF 20 µl
    g. Distill water to make 1 ml
12. Coommassie Brilliant Blue (CBB) Staining solutiom
    a. CBB R 250 2 gm
    b. Methanol 45 ml
    c. Glacial Acetic Acid 10 ml
    d. Distill water to make 100 ml
    Store the stain in tightly stoppered bottle. The staining solution may be reused several times.
13. Destaining solution
    a. Glacial Acetic Acid 20 ml
    b. Methanol 10 ml
    c. Distill water to make 100 ml
14. Gel casting glass plates, spacers and combs (for 15 cm X 15 cm X 0.6 mm gel)

PROCEDURE

A. PROTEIN SAMPLE PREPARATION

Take the tissue/cells which are to be used as the source of protein and put them in the sample buffer (volume depends on the amount of protein in the tissue) in a 1.5 ml Eppendorf tube, close the tube tightly and immediately keep the tube in a boiling water-bath for 5-10 min. Make sure that the tube cap does not open in between. After 10 min, briefly spin the tube and load the sample in the gel or store the sample at -70 ^{0}C till use.

B. CASTING OF VERTICAL SLAB GEL

i. SEPERATING OF THE LOWER GEL

1) Assemble the cleaned glass plates with spacers of required thickness
2) For a 12.5 % separating polyacrylamide gel, prepare the following solution by adding in the order
   a. Distill water 3.46 ml
   b. 1 M Tris (pH 8.8) 7.50 ml
   c. Acrylamide Stock solution 8.36 ml
   d. 50 % Glycerol 0.40 ml
   e. 10 % SDS Stock solution 0.20 ml

   Mix, filter and degas for 5 min under vacuum (presence of dissolved oxygen inhibits polymerization of Acrylamide)

   Add the following polymerizing catalysts
   a. 10 % Ammonium persulphate 100 µl
   b. TEMED 10 µl

   Swirl the solution rapidly, taking care not to create any bubbles, and quickly proceed to the next step.
3) Pour the gel solution rapidly into the gap between the glass plates without trapping any bubbles. If any bubbles are trapped, they must be immediately removed either by tapping or by inserting a thin strip of plastic or X-ray film to dislodge the bubble. Leave sufficient space at the top for stacking gel (to be poured later).
4) Carefully overlay the separating gel acrylamide solution with water saturated iso-butanol (to prevent contact of the acrylamide solution with atmospheric oxygen).
5) Leave the gel mold undisturbed for 45-60 min to let the acrylamide polymerize. Polymerization is complete when the interface between the gel and the overlaid water becomes distinct.
6) When the polymerization is complete, drain off the overlaid water, wash the top layer several times, with distilled water to remove any unpolymerized acrylamide. Drain the excess water completely.

ii. STACKING OF THE UPPER GEL

1) Prepare the following stacking gel (5 % acrylamide) solution
   a. Distill water 3.46 ml
   b. 1 M Tris (pH 6.8) 0.50 ml
   c. Acylamide stock solution 0.66 ml
   d. 10 % SDS 40 µl

   Mix and degas under vacuum for 5 min and add the TEMED and APS as follows
   e. 10 % APS 40 µl
   f. TEMED 4 µl

2) Quickly mix by swirling, without creating any air bubbles. Quickly pour the solution between the glass plates on top of the polymerized separating gel. Immediately insert the Teflon comb into the stacking gel (between the teeth of the comb and the separating gel) should not generally exceed 1 cm. Leave the gel assembly undisturbed for 45-60 min to let the stacking gel polymerize.
3) Carefully remove the comb and immediately wash the wells by flushing with distilled water using a syringe fitted with a fine hypodermic needle. After a thorough cleaning of the wells, the extending teeth of polymerized acrylamide are straightened, if required, with a blunt needle.

(Leaving the comb in the stacking gel for a longer period results in polymerization of acrylamide in the capillary space between the comb teeth and glass plates: this may lead to "streaks" in the fractionated protein bands. Therefore, the comb should be removed as soon as the gel has polymerized).

C. RUNNING OF GEL

1) Mount the gel assembly on the vertical gel tank and add the Tris-Glycine buffer to the upper and lower tanks. Care is taken to ensure that no bubbles are trapped in the gel wells.
2) Connect the wire leads from the gel tank to the power supply (the lower tank lead (red) is connected to the positive pole (anode) while the lead from the upper tank (black or blue) is connected to the cathode of the power supply).
3) Load the protein samples to be electrophoresed into the bottom of the well using a micro-syringe. (The actual volume of the sample loaded depends upon the dimensions of the well: it should not make a layer more than a few millimeters in height: for a 5 mm X 0.6 mm well, 20 µl of sample containing about 40 µg protein is adequate)
4) Apply current (20 mA constant for a 15 cm X 15 cm X 0.6 mm slab gel) till the dye enters the separating gel. At that time increase the current to 25 mA (constant) and run till the bromophenol dye front has reached bottom of the gel.
   (During the run, it is desirable that the temperature of the gel is maintained between 4-8 ^{o}C either by keeping the gel assembly in a cold room or, preferably, by circulating cold water in the gel assembly. (Gel tanks have special built in water circulating channels).
5) After the run is over, the gel assembly is disassembled and one of the glass plates is carefully removed. The orientation of the gel is marked by cutting one of the corners of the gel.

D. FIXING AND STAINING OF THE CELL

1) Remove the gel from supporting glass plate and carefully transfer it to a suitable size tray containing 5 volumes of the CBB staining solution. Leave overnight in the stain at room temperature.
2) Next morning, remove the stain and store for reuse. Add the gel de-staining solution. Replace the de-staining solution 3 times at 10-15 min intervals
3) After the final removal of de-staining solution, give two changes in 15 % acetic acid at 10 min intervals and finally leave the gel in 10 % acetic acid when the protein bands appear bright purple stained against an almost clear background.
4) The gel may be stored in 10 % acetic acid or may be dried before storage. If the proteins were labelled with radioactive precursors, the gel may be processed for autoradiography or fluorography as desired.

PRECAUTIONS

1. The gel assembly must remain free of any vibrations during the period of polymerization since vibrations cause unevenness in the polymerized matrix which results in distortions in protein bands. The table/platform on which the gel assembly is kept should be vibration free.
2. Gel must not get heated during run: this causes the typical “smiling” effect. Excessive heating may be prevented by regulating the current flow and by keeping the gel in a cold room or preferably by circulating cold water through the gel tank.
3. The wells must be thoroughly cleaned: any pieces of polymerized gel or other debris will result in distorted bands.
4. All the samples should be quickly loaded in respective wells to prevent diffusion of sample. While loading the samples, care must be taken to avoid any bubbles in the sample.

1.7. PROTEIN ESTIMATION BY LOWRY'S METHOD

INTRODUCTION

Protein can be estimated by different method as described by Lowry and also by estimating the total nitrogen content. No method is 100 % sensitive. Hydrolysing the protein and estimating the amino acids alone will give the exact quantification. The method developed by Lowry et al. is sensitive enough to give a moderately constant value and hence largely followed. Protein content of the enzyme extracts is usually determined by this method.

PRINCIPLE

The blue colour developed by the reduction of the phosphomolybdic-phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured in the Lowry's method.

MATERIALS REQUIRED

1. 2 % Sodium carbonate (Na_2CO_3) in 0.1 N NaOH (Reagent)
2. 0.5 % Copper Sulphate ($CuSO_4$. $5H_2O$) in 1 % potassium sodium tartrate (Reagent B)
3. Alkaline copper solution: mix 50 ml of A and 1 ml of B prior to use (Reagent C)
4. Folin-ciocalteau reagent (Reagent D): Reflux gently for 10 hours a mixture consisting of 100 gm tungstate ($Na_2WoO_4.2H_2O$), 25 gm sodium molybdate ($Na_2MoO_4.2H_2O$), 700 ml water, 50 ml pf 85 % phosphoric acid, and 100 ml of concentrated hydrochloric acid in a 1.5 l flask. Add 150 gm lithium sulphate, 50 ml water and a few drops of bromine water. Boil the mixture for 15 min without condenser to remove excess bromine. Cool, dilute to 1 l and filter. The reagent should have no greenish tint. (Determine the acid concentration of the reagent by titration with 1 N NaOH to phenolpthalein end-point)
5. Protein solution (Stock standard):
   Weigh accurately 50 mg of bovine serum albumin (Fraction V) and dissolve in distilled water and make up to 50 ml in a standard flask.
6. Working standard:
   Dilute 10 ml of the stock solution to 50 ml with distilled water in a standard flask. One ml of this solution contains 200 µg protein

PROCEDURE

Extraction of protein from sample

Extraction is usually carried out with buffers used for the enzyme assay. Weigh 500 mg of the sample and grind well with a pestle and mortar in 5-10 ml of the buffer. Centrifuge and use the supernatant for protein estimation.

Estimation of protein

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tubes.
2. Pipette out 0.1 ml and 0.2 ml of the sample extract in two other test tubes.
3. Make up the volume to 1 ml in all the test tubes. A tube with 1 ml of water serves as the blank.
4. Add 5 ml of reagent C to each tube including the blank. Mix well and allow to stand for 10 min.
5. Then add 0.5 ml of reagent D, mix well and incubate at room temperature in the dark for 30 min. Blue colour is developed.
6. Take the readings at 660 nm.
7. Draw a standard graph and calculate the amount of protein in the sample.

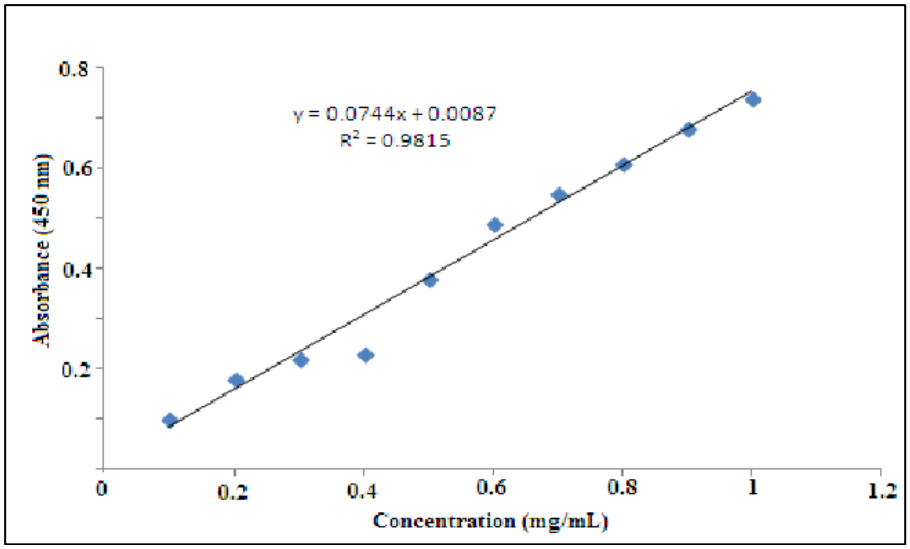


Figure: Sample standard curve for protein estimation by Lowry method

CALCULATION

Express the amount of protein mg/gm or 100 gm sample.

NOTES:

1. For complete enzyme extraction, sometimes the chemicals like ethylenediamine tetraacetic acid (EDTA), magnesium salts and mercaptoethanol are included. This method of protein estimation should not be followed if the extractant contains K^+, Mg^{++}, Tris EDTA and thiol (mercaptoethanol) compounds as they interfere with this procedure.
   When these chemicals are present in the extract, precipitate the protein by adding 10 % TCA, centrifuge and dissolve the precipitate in 2N NaOH and proceed for protein estimation.

2. If the protein concentration of the sample is high (above 500 µg/ml) measure the colour intensity at 550 nm.
3. Rapid mixing as the Folin reagent is added is important for reproducibility.
4. A set of standards is needed with each group of estimation preferably in duplicate. Duplicate or triplicate unknowns are also recommended.
5. Folin-ciocalteau reagent can be purchased commercially. Store refrigerated in amber bottles. A good quality reagent is straw yellow in colour.
6. If protein estimation is desired in a sample with high phenolic or pigment content, extract should be prepared with a reducing agent preferably cysteine and NaCl. Precipitate the protein with TCA, separate the protein and dissolve in 2N NaOH and proceed.

1.8. RNA ISOLATION FROM FUNGUS

RNA Isolation from fungal sample were done using NucleoSpin® RNA Plant and Fungi Kit from Macherey-Nagel.

INTRODUCTION

The NucleoSpin® RNA Plant and Fungi kit is designed for the isolation of RNA from diverse plant and fungal material, including samples rich in starch, sugar, secondary metabolites and other compounds that might interfere with common RNA isolation procedures.
First, the fungal material is mechanically disrupted (e.g., by NucleoSpin® Bead Tubes, grinding in liquid nitrogen, or any other suitable disruption method) in lysis buffer containing large amounts of chaotropic ions. This lysis buffer immediately inactivates RNases, which are present in virtually all biological materials. After removal of plant debris with the NucleoSpin® Plant and Fungi Filter, a binding solution is added which creates appropriate binding conditions which favor adsorption of RNA to the silica membrane. Washing steps with two different buffers remove salts, metabolites and macromolecular cellular components. Pure RNA is finally eluted under low ionic strength conditions with RNase-free water.
The RNA preparation using NucleoSpin® RNA Plant and Fungi can be performed at room temperature. The eluate, however, should be treated with care because RNA is very sensitive to trace contaminations of RNase, often found on general lab ware, fingerprints, and dust. To ensure RNA stability, keep RNA frozen at -20 °C for short-term or at -70 °C for long-term storage.

NOTES:

1. The kit is recommended for the isolation of RNA from diverse plant tissues and organs as well as filamentous fungi. The kit is not suitable for the isolation of small RNA (< 200 nt).
2. Typically, 50–500 mg sample input is recommended per preparation.

MATERIALS OR COMPONENTS

KIT COMPONENTS:

Reagent	Volume for 10 preparations	Volume for 50 preparations
Lysis Buffer PFL	8 ml	30 ml
Reduction Buffer PFR	5 ml	5 ml
Binding Buffer PFB	10 ml	45 ml
Wash Buffer PFW1	8 ml	30 ml

Wash Buffer PFW2 (concentrate) *	6 ml	25 ml
RNase-free H_2O	13 ml	13 ml
NucleoSpin® RNA Plant and Fungi Filter	10	50
NucleoSpin® RNA Plant and Fungi Columns (light blue rings – plus Collection Tube)	10	50
Collection Tubes (2 ml)	30	150
Collection Tubes (1.5 ml)	10	50

* **Wash Buffer PFW2**: Add the indicated volume of 96–100 % ethanol (see table below) to Wash Buffer PFW2. Mark the label of the bottle to indicate that ethanol was added. Wash Buffer PFW2 can be stored at room temperature (18–25 °C) for at least one year.

Reagent	Volume for 10 preparations	Volume for 50 preparations
Wash Buffer PFW2 (concentrate)	6 ml Add 24 ml ethanol	12 ml Add 48 ml ethanol

OTHER REQUIREMENTS

Reagents
• 96–100 % ethanol (for preparation of Buffer PFW2)

Consumables
• Disposable pipette tips

Equipment
• Manual pipettes
• Centrifuge for microcentrifuge tubes
• Equipment for sample disruption and homogenization (mortar, pestle, liquid N_2)
• Personal protection equipment (lab coat, gloves, goggles)

METHODOLOGY

Before starting the RNA isolation process, the buffer volumes required for the specific amount of samples

Sample Type	Sample amount per preparation	Buffer PFR	Buffer PFB
Fungal hyphae	50 mg	20 µl	750 µl
Fungal fruiting body	50-100 mg	10 µl	500 µl

1. **Homogenizing the sample**
    a. Add 500 µl buffer PFL into a 1.5- or 2-ml microcentrifuge tube (not provided).
    b. Add 10-50 µl buffer PFR
    c. Precool mortar and pestle at -70 °C in a freezer.
    d. Add into the mortar containing liquid nitrogen.
    e. Grind the sample with liquid N_2 until a fine powder is obtained.
    f. Transfer sample to the Buffer PFL/PFR mixture and mix immediately. The plant material shall only thaw within the lysis buffer.
    g. Incubate lysis tube for 5 min at 56 °C.

    Note:
    Centrifuge for 1 min at 14,000 x g in order to sediment cell debris.
    Note: If the cell debris pellet is not sufficiently solid, centrifuge for a longer time (e.g., 3min) and/or at 20,000 x g.
    Continue with the clear supernatant.

2. **Filtrating Lysate**
    a. Insert a NucleoSpin® RNA Plant and Fungi Filter Column (green ring) into a Collection Tube (2 ml, provided).

    Note:
    Alternatively use a 2 ml microcentrifuge tube with lid (not provided). This facilitates mixing by vortexing in step 3

    b. Load the clear lysate from step 1 onto the column.
    c. Centrifuge for 1 min at 14,000 x g.

    Note:
    In some cases, a small pellet will form. This pellet does not have to be removed and can be processed together with the supernatant.

    Note:
    If the sample does not pass the column completely, centrifuge at 20,000 x g for additional 3 min.

3. **Adjusting RNA binding conditions**
    a. Add 500 µl Buffer PFB to the flow-through and mix by pipetting.
    b. Incubate for 5 min at room temperature.

4. **Binding RNA**
    a. For each preparation take one NucleoSpin® RNA Plant and Fungi Column (light blue ring) preassembled with a Collection Tube.
    b. Load 650 µl of the sample onto the NucleoSpin® RNA Plant and Fungi column.
    c. Centrifuge for 30 s at 14,000 x g.
    d. Discard the flow-through and reuse the collection tube.
    e. Load the residual sample volume (approx. 200 µl) onto the column.
    f. Centrifuge for 30 s at 14,000 x g.
    g. Discard collection tube with flow-through and insert the column into a fresh Collection Tube (2 ml, provided).

5. **Washing and drying silica membrane**
    a. 1st Wash
        i. Add 500 µl Buffer PFW1 onto the column.
        ii. Centrifuge for 1 min at 14,000 x g.
        iii. Discard collection tube with flow-through and insert column into a fresh Collection Tube (2 ml, provided).
    b. 2nd Wash
        i. Add 500 µl Buffer PFW2 onto the column.
        ii. Centrifuge for 1 min at 14,000 x g.
        iii. Discard flow-through and discard collection tube unless the following additional wash step is included.
    c. 3rd Wash
        i. Add 500 µl Wash Buffer PFW2 onto the column.
        ii. Centrifuge or 1 min at 14,000 x g.
        iii. Discard collection tube with flow-through.

6. **Elution of RNA**
    a. Insert column into a fresh Collection Tube (1.5 ml, provided).
    b. Add 50 µl RNase-free H_2O onto the column.
    c. Incubate for approximately 1 min at room temperature.
    d. Centrifuge for 1 min at 14,000 x g.

    Note:
    If higher RNA concentrations are desired, elution can be done with 40 µl. Overall yield, however, will decrease when using smaller volumes.

7. **Quantification of RNA**
    a. Bring RNA samples as well as the water used to elute them on ice to the spectrophotometer
    b. Wash the sample reader with molecular grade water and dry with a tissue

c. Following the software's instructions, load 2 µl of elution water (blank) and initialize the system
d. Change the computer's setting to RNA and click the blank button
e. Load 2 µl of sample and click the measure button
f. After the read is complete, record the A260/A280 and A260/A230 ratios as well as the amount of RNA recovered (in ng/µl)
g. Wipe the sample reader with a clean, dry tissue between samples and repeat steps

Note

- Very pure RNA will have an A260/A280 ratio of ~2.1. Anything higher than 1.8 is considered to be of acceptable purity, and a ratio of <1.8 indicates potential DNA or protein contamination. A low A260/A280 ratio is likely due to mixing phases when removing the upper aqueous phase of the Trizol separation or is also more common in samples with a very low yield of RNA.
- The A260/A230 ratio should also be above 2.0. A low A260/230 ratio indicates contamination with the wash solutions, chaotropic salts, phenols or protein. A low A260/A230 ratio is most likely due to contamination of the samples with washing buffers. Be more careful when handling the tubes, especially when adding wash solution or removing the spin-through. Try to gently pour out the flow-through and then carefully wipe away drops on the outer rim of the collection tube with a dry tissue.

Note:
RNA concentration, retrieved, is usually 100-200 ng/ µl

1.9. cDNA SYNTHESIS

The cDNA synthesis has been based on the BIORAD iScript™ cDNA Synthesis Kit (Catalog 1708890)

INTRODUCTION

iScript cDNA Synthesis Kit provides a sensitive and easy-to-use solution for two-step reverse transcription quantitative PCR (RT-qPCR). All the reagents provided are mentioned later.
The iScript Reverse Transcriptase is RNase H+, which provides greater sensitivity than RNase H- enzymes in qPCR. iScript is a modified Moloney murine leukemia virus (MMLV) reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA. The enzyme is provided preblended with RNase inhibitor. The unique blend of oligo(dT) and randomhexamer primers in the iScript Reaction Mix works exceptionally well with a wide variety of targets. This blend is optimized for the production of targets <1 kb in length. iScript cDNA Synthesis Kit produces excellent results in both real-time and standard RT-qPCR.

Storage and Stability

To be stored at -20 ˚C, with a guarantee for 12 months in a constant temperature freezer. Nuclease free water can be stored at room temperature.

KIT CONTENTS

Reagent	Volume for 25 reactions	Volume for 100 reactions
5x iScript Reaction Mix	100 µl	400 µl
iScript Reverse Transcriptase	25 µl	100 µl
Nuclease-free water	1.5 ml	1.5 ml

REACTION SETUP

The 5x iScript Reaction Mix may generate some precipitation upon thawing; this does not affect the quality of the mixture. If you do experience precipitation, please mix thoroughly to resuspend and use as directed in the following table.

Components

Component	Volume per reaction, µl
5x iScript Reaction Mix	4
iScript Reverse Transcriptase	1

Nuclease-free water	10
RNA template (100 fg-1 µg total RNA) *	5
Total volume	20

*When using larger amounts of input RNA (>1 µg), the reaction should be scaled up (for example, 40 µl reaction for 2 µg, or 100 µl reaction for 5 µg) to ensure optimum synthesis efficiency

REACTION PROTOCOL

Incubate the complete reaction mix in a thermal cycle using the following protocol:

Priming	5 min at 25 ˚C
Reverse transcription	20 min at 46 ˚C
RT inactivation	1 min at 95 ˚C
Optional step	Hold at 4 ˚C

Recommendation for Optimal Results using the iScript cDNA Synthesis Kit

The maximum amount of the cDNA reaction that is recommended for downstream PCR is one-tenth of the reaction volume, typically 2 µl.

1.10. PROCEDURE FOR AGAROSE GEL ELECTROPHORESIS

INTRODUCTION

The standard method used to separate and identify DNA fragments is electrophoresis through agarose gels. The location of DNA within the gel can be determined directly by visualizing ethidium bromide-stained fluorescent bands in ultraviolet light (254 nm or 310 nm or 354 nm) using a transilluminator.

The electrophoretic mobility of DNA through agarose gel is dependent on the following 4 parameters:

a. Molecular size of DNA
b. Agarose concentration
c. Confirmation of the DNA
d. Applied current

MATERIALS REQUIRED

A. Solutions

1. Tris-Acetate-EDTA (TAE) stock solution (10 X)
   a. Tris Base 48.5 gm
   b. Glacial Acetic Acid 11.4 ml
   c. 0.5 (M) EDTA (pH 8.0) 20.0 ml
   d. Distilled water to make 1000 ml

2. Working Buffer: 1 X

3. Loading Buffer (10 X)
   a. 0.25 % Bromophenol Blue
   b. 0.25 % Xylene cyanol
   c. 25 % Ficoll (Type 400) in distilled water
   Store it in room temperature

B. Preparation of Agarose Gel

For Analyzing restricted digested genomic DNA, usually a 0.8 % gel is made in 0.5 % or 1 X TAE (a higher or lower % gel can be used depending upon the size of DNA molecules to be analyzed)

1. Prepare the gel mold by sealing the two free ends of gel platform with plastic tape and positioning the comb on the gel platform (at least 0.5-0.1 mm gap should be left between the platform and base of the comb, otherwise wells may get damaged)
2. Add the appropriate amount of agarose to a measured quantity of the electrophoresis buffer (e.g., for a medium size gel, take 320 mg of agarose in 40 ml of 0.5 X TAE)
3. Boil till the agarose dissolves (total of about 10 min heating on a heater)

4. Cool solution to 50 ^{o}C
5. Pour the gel solution into the prepared mold; let it cool at room temperature (avoid any vibrations to the table or the gel mold while polymerization is in progress)
6. After the gel is completely set (30-45 min at room temperature), carefully remove the comb (preferably add electrophoretic buffer beforehand so that the comb comes off easily)
7. Remove the plastic tape from both ends of geld mold and put the gel with its platform in a submarine electrophoresis. Add enough electrophoresis buffer (1 X or 0.5 X TAE) to let the gel just submerge.
8. Prepare DNA samples for electrophoresis as follows
    a. DNA (at least 150-200 ng) + distilled water : 18 µl
    b. 10 X Loading buffer : 2 µl

    (N.B. Volume of water can be adjusted according to the volume of DNA sample)
9. Vortex the samples and centrifuge briefly before loading in the wells. Switch on the power supply at 50-150 volts depending upon the gel size (5 volts/cm being the optimal).
10. After the run is over, stain the gel with EtBr dye and shook in a gel-rocker for 15-20 mins.
11. Examine the gel in ultra-violet light using a transilluminator prior to watching it in gel-doc, for visibility of bands and photo-documentation.

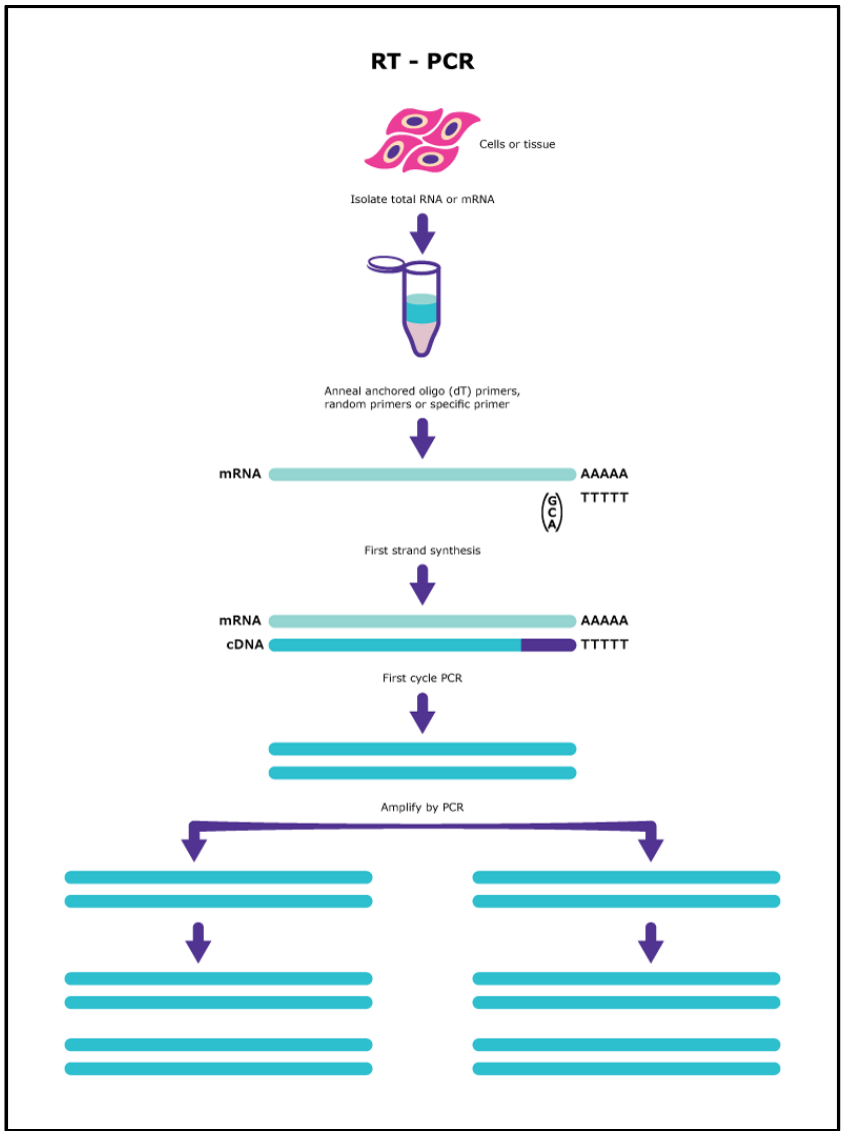
1.11. PCR AMPLIFICATION OF cDNA FOR EXPRESSION PROFILING

INTRODUCTION

Reverse transcription polymerase chain reaction (RT-PCR) is a variation of **standard PCR** that involves the amplification of specific mRNA obtained from small samples. It eliminates the need for the tedious mRNA purification process required for conventional cloning techniques. In RT-PCR, reverse transcriptase and an RNA sample are used in addition to the standard PCR reagents. The reaction mixture is heated to 37 ˚C, which enables the production of cDNA from the RNA sample by reverse transcription. This cDNA anneals to one of the primers leading to first-strand synthesis. Standard PCR proceeds and dsDNA is produced.
The primers used must match the project. If amplification of all of a cell's mRNA is required, oligo (dT) primers are sufficient because they anneal to the poly(A) tails. If a specific mRNA is to be amplified, a coding region-specific primer can be used.

Standard PCR

The polymerase chain reaction or PCR is a powerful technique that allows amplification of a specific DNA sequence in the genome. The basic principles of PCR rests on the fact that a DNA polymerase, to replicate the parental DNA needs a short primer sequence that is complementary to a region in the template. Since all DNA polymerases elongate the nascent chain 5' to 3' direction, a given stretch of DNA is replicated in both directions using two different primers. In case of PCR, a specific set of two primers, each complementary to short sequences on either sides of the region that is to be amplified are allowed to anneal and a DNA polymerase then elongates primers on both ends to replicate the intervening region; this cycle of denaturation of the template DNA, reannealing with primers and chain elongation is repeated several times so that the yield of the newly synthesized DNA corresponding to a very specific region is substantial. Hence the name polymerase chain reaction. This principle could be put in actual wise with the discovery of a thermostable DNA polymerase isolated from a thermophilus bacteria, *Thermophilus aquaticus*. This enzyme withstands high temperature required for denaturation of the template and primer DNAs. Thus, the same reaction mix can be allowed to go through multiple rounds of denaturation, annealing and chain elongation for exponential increase in the yield of target DNA. In view of its sensitivity, PCR has manifold applications in molecular biology, genetic engineering and DNA fingerprinting.



The following gives a typical protocol for PCR amplification of xylanase gene using XYL2 primers.

MATERIALS REQUIRED

1. Purified fungal cDNAs (concentration of 15-50 ng/µl)
2. Primers (10 pM/µl stock solution of each primer)
   F: 5'>GCTCAATCGGACAAACCAAT<'3
   R: 5'>GTCGGTGATGGTTCCGTAGT<'3
3. DNA polymerase (Primestar)
4. Thermal cycler or Three Water baths set at 92 ^{o}C, 72 ^{o}C and 60 ^{o}C, respectively.
5. 1 % Agarose gel for fractionation of PCR product and related units and buffers (mentioned in a protocol earlier) and Gel documentation unit
6. Fresh and sterilized tips and tubes.

PROCEDURE

1. Prepare the agarose gel as per the procedure mentioned earlier
2. For the sample preparation:
   Using fresh autoclaved or sterile tips, add the following to an absolutely clean PCR tube
   a. Nuclease free H_2O 2 µl
   b. Primestar (DNA polymerase) 5 µl
   c. Forward primer (prepared earlier) 1 µl
   d. Reverse primer (prepared earlier) 1 µl
   e. cDNA template (prepared earlier) 1 µl

   Total 10 µl
   Mix the contents in the PCR tube
3. Then in the thermal cycler, repeat the following steps:
   a. Denature the contents by keeping the tube at 96 ^{o}C for 6 mins.
   b. Repeat the following steps for 32 cycles*
      i. at 98 ^{o}C for 30 seconds
      ii. at 55 ^{o}C for 20 seconds
      iii. at 72 ^{o}C for 45 seconds
   c. at 72 ^{o}C for 3 mins
   d. at 12 ^{o}C for 15 mins
   e. Hold at 4 ^{o}C for infinite
4. After the PCR is ran, the PCR product is vortexed and centrifuge briefly before loading in the wells. Switch on the power supply at 50-150 volts depending upon the gel size (5 volts/cm being the optimal). Run for agarose gel electrophoresis as mentioned earlier.
5. After the run is over, stain the gel with EtBr (Ethidium Bromide) dye and shaken in a gel-rocker for 15-20 mins.
6. Examine the gel in ultra-violet light using a transilluminator prior to watching it in gel-doc, for visibility of bands and photo-documentation.

*For the gene expression analysis, the cycles of the PCR were varied, for optimization purposes, which would be observable by the band width and intensity.

PRECAUTIONS

1. Extreme care is to be taken care with cleanliness of all tubes, tips and solutions: any trace of contaminating DNA may also be amplified and cause spurious results.
2. The annealing temperature condition needs to be varied depending upon the nature of primers.

1.12. PRIMERS USED

	Primer sequence F/R [5'-3'] (F=Forward; R=Reverse)	Amplicon length [bp]	Amplicon Temperature Tm [°C]	Total Yield (µg)	Volume (µl) to be assed to get 100 pm/ µl or 100 µM conc.)	Concentration (pm/µl)
Xylanase 2 (XYL2)	F: 5'>GCTCAATCGGACAAACCAAT<'3	20	T_m=50	251.12 (MW=6.079kDa)	413.10	206.55
	R: 5'>GTCGGTGATGGTTCCGTAGT<'3	20	T_m=54	270.08 (MW=6.195kDa)	435.98	217.99
Xylanase 3 (XYL3)	F: 5'>ATCTTCGCTGAGGACGGTAA<'3	20	T_m=52	259.53 (MW=6.157kDa)	421.52	210.76
	R: 5'>GGCAAGCGTTCATGACAGTA<'3	20	T_m=52	265.75 (MW=6.166kDa)	431.00	215.50
Xylanase 5 (XYL5)	F: 5'>TGGTAAGGGTTGGTCTCCTG<'3	20	T_m=54	264.09 (MW=6.194kDa)	426.30	213.15
	R: 5'>GCTGGAAGGTCTGAGTACCG<'3	20	T_m=56	263.58 (MW=6.198kDa)	425.58	212.64

A, T, G, C – are the usual representation of nucleotide base pairs

Primer3 (v. 0.4.0) Pick primers from a DNA sequence.	Checks for mispriming in template.	disclaimer	Primer3 Home
	Primer3plus interface	cautions	FAQ/WIKI

There is a newer version of Primer3 available at http://primer3.ut.ee

Paste source sequence below (5'->3', string of ACGTNacgtn -- other letters treated as N -- numbers and blanks ignored). FASTA format ok. Please N-out undesirable sequence (vector, ALUs, LINEs, etc.) or use a Mispriming Library (repeat library): NONE

```
TAACTGCTTACTCGGTCGTTGGAAGATAGCTGGGCTAGTAGTCAATACATACCCTTACCATTAAAACATC
TGATCTCTGTGATTCACCTGCCCTCGATGTCTCAATTAATGTAGTATAATGCACTTTCGGCAAGACTAAT
GAGTGAGATAAAAGTAGCATGTGTGAAACTAGTCCTTCAGATGAGAGTGTCACTAACGGTGCCCAGGTGC
AGTGACGTTCAAGACTCAGCCGTAGTCAAAAGACATCCGATTCAGGCTGCTGTAACGCCGGTAGTTGAAT
ATGGCGTGGCCGTCCGCCAAGATCTTGTCGTGATAGCGGACGAGAAATTCCGGCAGACATTGTTTGCTTC
TGGATCGCGGTAGCCAACCGGCAAGTATTCGACAATCTGCCCGGAGCTGCAGTGCAC
```

Pick left primer, or use left primer below:	Pick hybridization probe (internal oligo), or use oligo below:	Pick right primer, or use right primer below (5' to 3' on opposite strand):

Pick Primers Reset Form

Sequence Id: A string to identify your output.

Targets: E.g. 50,2 requires primers to surround the 2 bases at positions 50 and 51. Or mark the source sequence with [and]: e.g. ...ATCT[CCCC]TCAT.. means that primers must flank the central CCCC.

Excluded Regions: E.g. 401,7 68,3 forbids selection of primers in the 7 bases starting at 401 and the 3 bases at 68. Or mark the source sequence with < and >: e.g. ...ATCT<CCCC>TCAT.. forbids primers in the central CCCC.

Product Size Ranges 301-400

Number To Return 5 Max 3' Stability 9.0

Max Repeat Mispriming 12.00 Pair Max Repeat Mispriming 24.00

Max Template Mispriming 12.00 Pair Max Template Mispriming 24.00

Pick Primers Reset Form

General Primer Picking Conditions

	Min:	Opt:	Max:		
Primer Size	17	20	27		
Primer Tm	57.0	60.0	60.0	Max Tm Difference: 100.0	Table of thermodynamic parameters: Breslauer et al. 1986
Product Tm					
Primer GC%	40.0		60.0		

Max Self Complementarity: 8.00 Max 3' Self Complementarity: 3.00

xl

Max #N's:	0	Max Poly-X:	5	
Inside Target Penalty:		Outside Target Penalty:	0	Note: you can set Inside Target Penalty to allow primers inside a target.
First Base Index:	1	CG Clamp:	0	
Concentration of monovalent cations:	50.0	Salt correction formula:	Schildkraut and Lifson 1965	
Concentration of divalent cations	0.0	Concentration of dNTPs	0.0	
Annealing Oligo Concentration:	50.0	(Not the concentration of oligos in the reaction mix but of those annealing to template.)		

☑ Liberal Base ☐ Show Debuging Info ☑ Do not treat ambiguity codes in libraries as consensus ☐ Lowercase masking

Pick Primers Reset Form

Other Per-Sequence Inputs

Included Region:		E.g. 20,400: only pick primers in the 400 base region starting at position 20. Or use { and } in the source sequence to mark the beginning and end of the included region: e.g. in ATC{TTC...TCT}AT the included region is TTC...TCT.
Start Codon Position:		

Sequence Quality

Min Sequence Quality:	0	Min End Sequence Quality:	0	Sequence Quality Range Min:	0	Sequence Quality Range Max:	100

Objective Function Penalty Weights for Primers

Tm	Lt:	1.0	Gt:	1.0
Size	Lt:	1.0	Gt:	1.0
GC%	Lt:	0.0	Gt:	0.0

Self Complementarity	0.0
3' Self Complementarity	0.0
#N's	0.0
Mispriming	0.0
Sequence Quality	0.0
End Sequence Quality	0.0
Position Penalty	0.0
End Stability	0.0
Template Mispriming	0.0

Objective Function Penalty Weights for Primer *Pairs*

Product Size	Lt:	0.0	Gt:	0.0

Product Tm Lt: 0.0 Gt: 0.0

Tm Difference 0.0

Any Complementarity 0.0

3' Complementarity 0.0

Pair Mispriming 0.0

Primer Penalty Weight 1.0

Hyb Oligo Penalty Weight 0.0

Primer Pair Template Mispriming Weight 0.0

Hyb Oligo (Internal Oligo) Per-Sequence Inputs

Hyb Oligo Excluded Region:

Hyb Oligo (Internal Oligo) General Conditions

Hyb Oligo Size: Min 18 Opt 20 Max 27

Hyb Oligo Tm: Min 57.0 Opt 60.0 Max 63.0

Hyb Oligo GC% Min: 20.0 Opt: Max: 80.0

Hyb Oligo Self Complementarity:	12.00	Hyb Oligo Max 3' Self Complementarity:	12.00
Max #Ns:	0	Hyb Oligo Max Poly-X:	5
Hyb Oligo Mishyb Library:	NONE	Hyb Oligo Max Mishyb:	12.00
Hyb Oligo Min Sequence Quality:	0		
Hyb Oligo Conc of monovalent cations:	50.0	Hyb Oligo DNA Concentration:	50.0
Hyb Oligo conc of divalent cations:	0.0	Hyb Oligo [dNTP]	0.0

Objective Function Penalty Weights for Hyb Oligos (Internal Oligos)

Hyb Oligo Tm Lt: 1.0 Gt: 1.0

Hyb Oligo Size Lt: 1.0 Gt: 1.0

Hyb Oligo GC% Lt: 0.0 Gt: 0.0

Hyb Oligo Self Complementarity 0.0

Hyb Oligo #N's 0.0

Hyb Oligo Mishybing 0.0

Hyb Oligo Sequence Quality 0.0

Pick Primers Reset Form

Copyright Notice and Disclaimer

Citing Primer3

We request that use of this software be cited in publications as

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3 - new capabilities and interfaces. *Nucleic Acids Research* 40(15):e115
Koressaar T, Remm M (2007) Enhancements and modifications of primer design program Primer3 *Bioinformatics* 23(10):1289-91

Source code available at primer3.sourceforge.net/.

Acknowledgments

The development of Primer3 and the Primer3 web site was funded by Howard Hughes Medical Institute and by the National Institutes of Health, National Human Genome Research Institute, under grants R01-HG00257 (to David C. Page) and P50-HG00098 (to Eric S. Lander).

We thank Centerline Software, Inc., for use of their TestCenter memory-error, -leak, and test-coverage checker.

Primer3 was a complete re-implementation of an earlier program: Primer 0.5 (*Steve Lincoln, Mark Daly, and Eric S. Lander*). *Lincoln Stein* championed the idea of making Primer3 a software component suitable for high-throughput primer design.
Web interface by *Steve Rozen*

`(input.htm v 0.4.0)`

Last updated: $Id: index.html,v 1.1 2012/11/20 06:59:16 root Exp $$

Primer3 Output

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len       tm      gc%   any   3'  seq
LEFT PRIMER         47   20    59.94    45.00  3.00  2.00 GCTCAATCGGACAAACCAAT
RIGHT PRIMER       435   20    59.85    55.00  3.00  1.00 GTCGGTGATGGTTCCGTAGT
SEQUENCE SIZE: 1877
INCLUDED REGION SIZE: 1877

PRODUCT SIZE: 389, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 1.00

    1 ACAGCCCTAGTACTCGGAGCACCGTCCGAATACTGAGAAGGGGCTAGCTCAATCGGACAA
                                                  >>>>>>>>>>>>>>

   61 ACCAATCCCGGGCATTGACTCTTTCGAGAAAACTATCGTACAGCTATAGAGCAAGGGACT
      >>>>>>

  121 GACATGATCAAGATGTATAAGATGATTTAGCCCACCTTCTCTTGGCACTTGGCATTCTCA

  181 TCACTCAAACAACTACATTCATTACAACACCTTCACAGCTTCATTCATCGTTTGCAGGTC

  241 AATCAAACGTTTTATCTTTCTTTGTCACATATCTCTACATCAAGAACTTCTTCCAAAATG

  301 AAGCTGTCTTCCTTCCTCTACACCGCCTCGCTGGTCGCGGCCATTCCCACCGCCATCGAG

  361 CCCCGCCAGGCCTCCGACAGCATCAACAAGCTGATCAAGAACAAGGGCAAGCTCTACTAC
                                                              <<<<<

  421 GGAACCATCACCGACCCCAACCTGCTCGGCGTCGCAAAGGACACTGCCATCATCAAGGCT
      <<<<<<<<<<<<<<<

  481 GACTTTGGCGCCGTCACACCCGAAAATTCCATGAAGTGGGATGCCACCGAGCCCAGCCAG

  541 GGCAAGTTCAACTTCGGCAGCTTCGACCAGGTCGTCAACTTTGCTCAGCAGAATGGCCTC

  601 AAGGTCCGAGGTCACACTGTAGTCTGGCACTCCCAGCTCCCTCAGTGGGTTAAGAACATC

  661 AACGACAAGGCTACTTTGACCAAGGTCATCGAGAACCACGTCACCAACGTCGTTGGACGC

  721 TACAAGGGCAAGATCTACGCCTGGGTATGTTTTCTTCACTCGAACTTCTTATAAATGGCT

  781 TTACTAACATGTTCAGGACGTCGTTAACGAGATCTTCGACTGGGATGGTACCCTCCGAAA

  841 GGACTCTCACTTCAACAACGTCTTCGGCAACGACGACTACGTTGGCATTGCCTTCCGCGC
```

```
   901 TGCCCGCAAGGCTGACCCCAACGCCAAGCTGTACATCAACGACTACAGCCTCGACTCCGG

   961 CAGCGCCTCCAAGGTCACCAAGGGCATGGTTCCCTCTGTCAAGAAGTGGCTCAGCCAGGG

  1021 CGTCCCCGTCGACGGTATTGGTTCTCAGACTCACCTTGACCCCGGTGCCGCTGGCCAAAT

  1081 CCAGGGTGCTCTCACTGCCCTCGCCAACTCTGGTGTGAAGGAGGTTGCCATCACCGAGCT

  1141 CGACATCCGCACTGCCCCCGCCAACGACTACGCTACCGTTACCAAGGCCTGCCTCAACGT

  1201 CCCCAAGTGCATTGGTATCACCGTCTGGGGCGTATCTGACAAGAACTCTTGGCGCAAGGA

  1261 GCACGACAGCCTTCTGTTCGATGCTAACTACAACCCCAAGGCTGCTTACACTGCTGTTGT

  1321 CAACGCTCTCCGCTAAATGTGGCTTGCGTGACAGGATCAGGTCTCTGGTCTTGAGGGGGA

  1381 GCATCGTTGCAGTTGCTTTTGGTCATCACCGTATTCTTCTTTTATAACTCTGTATATAGT

  1441 TGTTTGTTTGTACAAAATCTCGACAAATGGTAACTGCTTACTCGGTCGTTGGAAGATAGC

  1501 TGGGCTAGTAGTCAATACATACCCTTACCATTAAAACATCTGATCTCTGTGATTCACCTG

  1561 CCCTCGATGTCTCAATTAATGTAGTATAATGCACTTTCGGCAAGACTAATGAGTGAGATA

  1621 AAAGTAGCATGTGTGAAACTAGTCCTTCAGATGAGAGTGTCACTAACGGTGCCCAGGTGC

  1681 AGTGACGTTCAAGACTCAGCCGTAGTCAAAAGACATCCGATTCAGGCTGCTGTAACGCCG

  1741 GTAGTTGAATATGGCGTGGCCGTCCGCCAAGATCTTGTCGTGATAGCGGACGAGAAATTC

  1801 CGGCAGACATTGTTTGCTTCTGGATCGCGGTAGCCAACCGGCAAGTATTCGACAATCTGC

  1861 CCGGAGCTGCAGTGCAC
```

KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

ADDITIONAL OLIGOS

	start	len	tm	gc%	any	3'	seq
1 LEFT PRIMER	923	20	59.87	50.00	6.00	1.00	GCCAAGCTGTACATCAACGA
RIGHT PRIMER	1316	20	59.81	55.00	3.00	1.00	CAGCAGTGTAAGCAGCCTTG

```
    PRODUCT SIZE: 394, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 2.00

  2 LEFT PRIMER        493   20   59.65   45.00  4.00  2.00 GTCACACCCGAAAATTCCAT
    RIGHT PRIMER       832   20   59.93   55.00  6.00  1.00 GGTACCATCCCAGTCGAAGA
    PRODUCT SIZE: 340, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 1.00

  3 LEFT PRIMER        505   20   59.76   45.00  4.00  2.00 AATTCCATGAAGTGGGATGC
    RIGHT PRIMER       825   20   59.80   50.00  6.00  2.00 TCCCAGTCGAAGATCTCGTT
    PRODUCT SIZE: 321, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 1.00

  4 LEFT PRIMER         47   20   59.94   45.00  3.00  2.00 GCTCAATCGGACAAACCAAT
    RIGHT PRIMER       415   20   59.62   50.00  4.00  0.00 AGAGCTTGCCCTTGTTCTTG
    PRODUCT SIZE: 369, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 3.00

 Statistics
          con   too    in    in          no    tm    tm  high  high        high
          sid  many   tar  excl   bad    GC   too   too   any    3'  poly   end
        ered    Ns   get   reg   GC% clamp   low  high compl compl     X  stab    ok
 Left   16104     0     0     0  6976     0  2271  5707     0     3     0   154   993
 Right  16004     0     0     0  6490     0  2374  5949     1     5     0   168  1017
 Pair Stats:
 considered 793, unacceptable product size 775, high end compl 3, ok 15
 primer3 release 1.1.4


 (primer3_results.cgi release 0.4.0)
```

ANNEXURE II:

Reprints of Publications

Indian Journal of Weed Science **47**(1): 59–65, 2015

Molecular characterization and host range studies of indigenous fungus as prospective mycoherbicidal agent of water hyacinth

Writuparna Dutta, Durga Ray[1] and Puja Ray*
Department of Biological Sciences, Presidency University, Kolkata, West Bengal 700 073

Received: 3 January 2015; Revised: 8 March 2015

ABSTRACT

An indigenous fungal culture, isolated from diseased water hyacinth, in Bolpur, Santiniketan, West Bengal, India, was found to be causing severe blight and dieback disease on water hyacinth, under laboratory and field conditions. It was subjected to morphological and molecular characterization by amplification of 18S RNA gene fragment from genomic DNA using 18S gene universal primers. Subsequently with sequencing, GenBank database comparisons and phylogenetic analysis, the fungus was determined as *Alternaria japonica* Yoshii. Further the pathogen was evaluated for its host specificity to be developed as mycoherbicidal agent against this invasive weed. Host range of *A. japonica* was screened against 48 plant species in 42 genera representing 22 families in pot experiment. Water hyacinth was the only species strongly susceptible to spore suspension (5×10^5 conidia/ml) of *A. japonica*. Minor infection was observed on goosefoot which is not only a weed but also ecologically separated from water hyacinth. Thus, the use of this pathogen in the biological control of water hyacinth would be safe for plants of economic and ecological significance in India. The secondary metabolite produced by *A. japonica* was sprayed on the test plants but phytotoxic symptoms were produced on nine out of 48 plants tested, demonstrating that phytotoxin produced by the fungus is not host specific. Further field tests needs to ascertain its efficacy under more natural conditions.

Key words: *Alternaria japonica*, Biological control, Host specificity, Mycoherbicide, Water hyacinth

Water hyacinth, *(Eichhornia crassipes)*, a native of the tropical South America, is considered to be one of the most serious aquatic weeds (Holms *et al*. 1977). It has spread throughout tropical countries causing widespread problems to millions of users of aquatic bodies and its resources causing severe problems related to its use and management (Gopal 1987). Water hyacinth invasiveness has led to a tremendous negative impact on the social and economic conditions of the aquatic ecosystem, causing a global annual loss of more than US$ 100 million to hydroelectricity generation, irrigation schemes, fisheries, water transport, etc (Shabana 2005).

Various control mechanisms including, manual, mechanical, chemical and biological methods, have been implemented for preventing the invasiveness, or eradication of, water hyacinth by various workers with mixed results (Julien and Orapa 2001, Ray *et al*. 2008). Environmental concerns over the use of chemical herbicides (Ray *et al*. 2008) have drawn interest in biological control of macrophyte. In its native land, water hyacinth is attacked by a large complex of natural enemies including several arthropod agents and fungi (Bennett and Zwölfer 1972, Ray and Hill 2013). But in its range of introduction, in absence of control agents, water hyacinth also flourishes majestically. The biological control agents have provided excellent control of water hyacinth in many locations around the world including India (Center 1994, Coetzee *et al.* 2011).

Biological control of weeds using insects and pathogens has gained considerable importance over last five decades as they are eco- friendly, host specific and effective means of weed control. Among various biological control agents, several phytopathogenic fungi have been found effective against the weed (Charudattan 2001). Various studies have been done to develop these fungi associated with the water hyacinth as potential mycoherbicides (Ray and Hill 2013).

During the present study, a number of indigenous pathogens were isolated from water hyacinth from selected regions of West Bengal (W.B.), India. Among these, a culture of *Alternaria* Nees. (WHK-12), isolated from diseased water hyacinth, in Bolpur, Santiniketan, was observed as a promising mycoherbicidal candidate for biological

***Corresponding author:** puja.ray@gmail.com
[1]Department of Biomedicinal Science and Biotechnology, Paichai University, Daejeon 302-735, Republic of Korea

control of water hyacinth during previous studies (unpublished data). Mycoherbicidal potential of several other *Alternaria* species has been reported against water hyacinth from various parts of the world (Nag Raj and Ponnapa 1970, Shabana *et al.* 1997, Pathak and Kannan 2011). *Alternaria* species associated with the weed have also been known to cause severe blight followed by dieback disease to water hyacinth (Nag Raj and Ponnapa 1970, Ray and Hill 2012).

The next step after recovery and screening of potential biocontrol agents is its identification and host specificity test as there is always some risk involved in man's use of new substance or device, be it a drug, pesticide or an electronic device. The isolate was subjected to molecular characterization for identification up to species level. The host range testing schemes have been developed for assessing the safety of non-target plant species against the test pathogen (Wapshere 1974). Thus, an experiment was conducted to determine the host range of this test fungi (WHK-12) by observing the impact of its spore suspension and culture filtrate on 45 plant species in 42 genera representing 22 families.

MATERIALS AND METHODS

Isolation of fungus and culture preparation: The indigenous fungal strain was isolated and purified from the diseased leaves of water hyacinth collected from Bolpur, Santiniketan (Co-ordintes: 23.6700° N, 87.7200° E) in February 2014. The leaves showing disease symptoms were collected and put in large paper envelopes, brought to the laboratory for isolation of pathogens. Back at the laboratory, leaf pieces of about 2 mm^2 was cut from the margins of necrotic or chlorotic lesions on the surface. Pieces were then placed on earlier prepared petri-plates containing potato dextrose agar (PDA) medium and incubated for 3-4 days at 27°C. The fungal species isolated earlier was purified by streak-plate and sub culturing techniques. It was carried out until fresh true monocultures of the fungus were obtained. It was further mass cultured in modified Richard's broth according to Ray (2006) for the present study. The spores were obtained from fungal mat while the toxic filtrate was obtained from the metabolized broth after 21 days of incubation.

Morphological identification of fungus: The fungal strain isolated from infected *E. crassipes*, was morphologically identified by slide culture technique with Lactophenol as mounting medium and observed under Zeiss Axio Scope.A1 Microscope for morphological identification of the genus.

Molecular identification: To confirm the species of the fungi, the isolate was molecularly characterized. Genomic DNA from the fungal isolate mat was extracted by using genomic DNA Isolation Kit (Xcelgen). The DNA stock sample was then quantified using Nanodrop spectrophotometer (Thermo Fisher Scientific NanoDrop™ 1000 spectrophotometer). Purity of DNA was judged on the basis of optical density ratio at 260:280 nm. Concentration of DNA was estimated using the formula.

Concentration of DNA (mg/ml) = OD 260 x 50 x Dilution factor

Agarose 0.8% (w/v) in 0.5X TAE (pH 8.0) buffer was used for submarine gel electrophoresis. Ethidium bromide (1%) was added at 10 µl /100ml. The wells were charged with 5µl of DNA preparations mixed with 1 µl gel loading dye. Electrophoresis was carried out at 80V for 30 min at room temperature. DNA was visualized under UV using UV transilluminator. The DNA concentration and integrity were checked by electrophoresis of the sample on 0.8% agarose gel containing ethidium bromide. After electrophoresis, the agarose gel was photo-documented. 18S RNA gene fragment was amplified from the genomic DNA by PCR (Eppendorf Thermal Cycler), using 18S gene universal primers: 1F and 4R. Details of 18S universal primer sequences were as follows: 1F (CTGGTGCCAGCAGCCGCGGYAA) and 4R (CKRAGGGCATYACWGACCTGTTAT).

Amplified PCR product was then purified using Xcelgen Gel extraction kit, to remove contaminants. To confirm the targeted PCR amplification, 5 µl of PCR product from each tube was mixed with 1 µl of 6X gel loading dye and electrophoresed on 1.2 % agarose gel containing ethidium bromide (1 % solution at 10 ìl/100 ml) at constant 5V/cm for 30 min in 0.5 X TAE buffer. The amplified product was visualized as a single compact band of expected size (Approx 850bp) under UV light and documented by gel documentation system (Biorad). The concentration of the purified DNA was determined and was subjected to automated DNA sequencing BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Sequencing was carried out using BigDye® Terminator v3.1 Cycle sequencing kit. After cycling, the extension products were purified and mixed well in 10 µl of Hi-Di formamide. The contents were mixed on shaker for 30 minutes at 300xg. Eluted PCR products were placed in a sample plate and covered with the septa. Sample plate was heated at 95°C for 5 min, snap chilled and loaded into

autosampler of the instrument. Consensus sequence of 808 bp of 18S region was generated from forward and reverse sequence data using aligner software. The 18S region sequence generated was then used to carry out BLAST with the nr database of NCBI genbank database (http://www.ncbi.nlm.nih.gov; accessed: 17th Dec 2014). Based on maximum identity score, 15 sequences were selected for preparing the phylogenetic tree, constructed using MEGA 5.

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates (BP) are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 806 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

Host range studies of test pathogen: The plant species included in the host-range test were selected on the basis of their economic or ecological importance and their relation to the test pathogen or the target plant, water hyacinth. All the test plants taken were at their seedling or early growth stage. They were usually collected from the field during local survey and grown in plastic tubs or cups. All the pots were filled with soil fertilized with farm yard manure and 15-3-12 N:P:K, slow-release fertilizer. The aquatic plants used in the test were grown in similar plastic pots filled with water. For experimentation, plants were kept in the growth chamber conditions at 26 ^{o}C temperature and 75 to 85% relative humidity in plastic pots filled with sterilized soil.

Preparation of spore suspension and phytotoxic culture filtrate: Mass cultivation of the test fungi (WHK-12) was done in modified Richard's broth in ten Erlenmeyer flasks of capacity 1000 ml, each containing 700 ml Richard's broth. These flasks were incubated at 30 ^{o}C in Biological Oxygen Demand (BOD) incubator for 21 days, under condition of 12 hours of alternate day and light. After twenty-one days of incubation, the fungal mat was separated from the liquid metabolized broth, for obtaining spores. The fungal mat was crushed in sterile distilled water and filtered to obtain the spore suspension. Spore suspension (5 x 10^5 spores /ml) was prepared in sterilized distilled water and 0.01% Tween-20 using haemocytometer the metabolized broth was first filtered through eight layers of cheese-cloth then through Whatman No. 1 filter paper to obtain crude culture filtrate to test the phytotoxicity of the secondary metabolite.

Host specificity testing: The test plants were inoculated at the same time during the evening with the spore suspension of the fungi (WHK-12) at concentration 5 x 10^5 spores /ml. They were sprayed until runoff with spore suspension using atomizer. They were covered with transparent polythene bags to create a dew effect for 24 hours and placed in growth racks at 27^{o} C and about 75 to 80% relative humidity. The control plants were sprayed with sterile distilled water and 0.01% tween-20. Another set of similar test plants were sprayed with culture filtrate of the pathogen. All the treatments were replicated thrice. Disease intensity and severity was rated by visual observation at an interval of 24 hours for 30 days. Disease intensity was determined visually on the basis of initiation of disease and increase in disease area seven days after application of the inocula.

RESULTS AND DISCUSSION

Identification and confirmation of species

Morphologically, under microscope, pale brown, simple or branched conidiophores with catenulate conidia at the apex were observed. Section *jponicae* usually contains short to long, simple or occasionally branched primary conidiophores with a single conidiogenous locus (Woudenberg *et al.* 2013). Apical secondary conidiophores were seen to be produced with a single conidiogenous locus. Conidia were porosporous, acropetally developed, dark brown, cylindrical or spindle-shaped, often with cylindrical beaks. Conidia were short, to long-ovoid with transverse and longitudinal septa, conspicuously constricted at most of the transverse septa, in short chains. The species within this section also occur on *Brassicaceae*. Thus the fungal isolate was previously linked to the *A. brassicicola* species-group (Pryor and Gilbertson, 2000, Pryor and Bigelow 2003, Lawrence *et al.* 2013). But this association on being questioned by Hong *et al.* (2005) was later clustered in section *Japonicae* (Woudenberg *et al.* 2013). Further 18S RNA gene fragment was used for characterization and was amplified by PCR from genomic DNA using

18**S** gene universal primers. A single discrete PCR amplicon band of 850 bp was observed when resolved on agarose gel (Fig. 1). Consensus sequence of 808 bp of 18S region was generated (Fig. 2) from forward and reverse sequence data using aligner software. It was then used to carry out BLAST with the nr database of NCBI gene bank database and based on maximum identity score, 15 sequences were selected (Table 1) for preparing the phylogenetic tree, constructed using MEGA 5 (Fig. 3).

The phylogenetic tree is broadly divided into two main clades. The first clade comprises of *Ulocladium botrytis* strain UPSC 3539, *Alternaria cheiranthi* EGS 41-188, *A. alternata* strain HA4087, *A. alternata* strain SRC1lrK2f, *A. maritima* strain CBS 126.60, *A. alternata* isolate AFTOL-ID 1610, *A. alternata* strain FC007, *A. alternata* ATCC 28329, *A. alternata* AA6 and *A. alternata* strain S-f6. While the second clade shows that the closest to our fungal isolate is *A. japonica* strain HDJZZWM- 06 (with 99% identity and 4 BP). Among the same clade the next close ones are *Pleospora herbarum* strain CBS 191.86, *P herbarum*, NS3/NS4 region, *P. herbarum* ATCC 11681 and *P. herbarum* DAOM 150679 respectively. This shows that genetically, *A. japonica* is more closely related to *Pleospora* spp., while among the first clade shows the strains of *A. alternata* and its relatedness with *U. botrytis*. After all these analysis, itt was thus confirmed that the fungal strain isolated, to be similar to *Alternaria japonica* strain HDJZ-ZWM-06 (GenBank Accession Number: GQ354822.1) based on nucleotide homology and phylogenetic analysis.

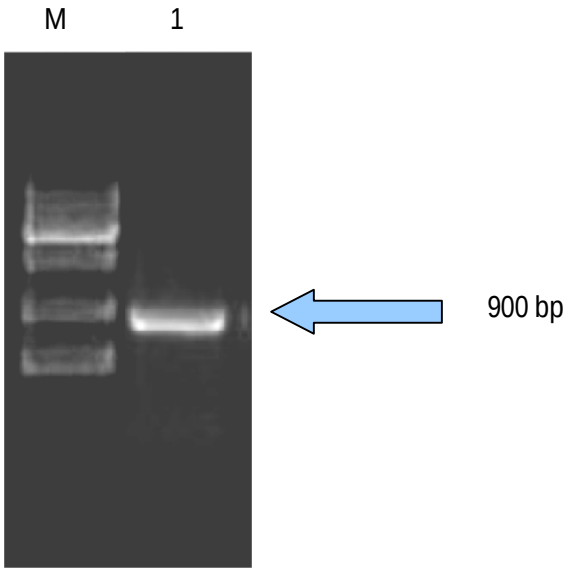


Fig. 1. 1.2% agarose gel image showing single 18S rDNA amplicon of 900 bp after purification by gel extraction. (Lane M: DNA marker (1kb ladder); Lane 1: 18S rDNA amplicon)

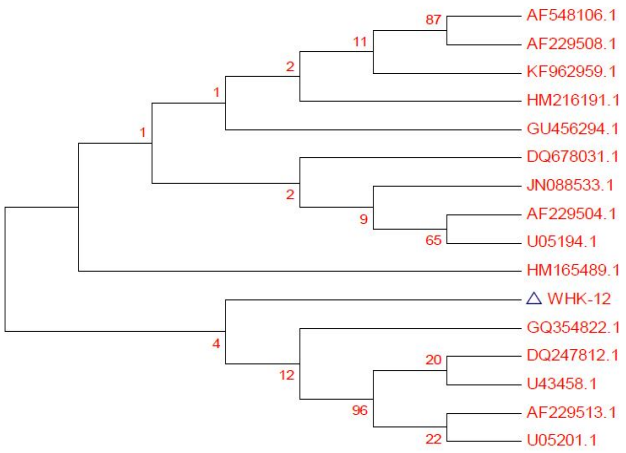


Fig. 3. Phylogenetic tree constructed from 15 closely related sequences, showing similarities between *Alternaria japonica* (WHK-12) and *A. japonica* strain HDJZ-ZWM-06 of Accession Number GQ354822.1. The tree was generated by using the Neighbor-Joining method using MEGA5.

```
TAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAAACTTGGGCCTGGCTGGCGGGTCCGCCTCACCGCGTGCACTC
GTCCGGCCGGGCCTTCCTTCTGAAGAACCTCATGCCCTTCACTGGGCGTGCTGGGGAATCAGGACTTTTACTTTG
AAAAAATTAGAGTGTTCAAAGCAGGCCTTTGCTCGAATACGTTAGCATGGAATAATAAAATAGGGCGTGCGTTTC
TATTTTGTTGGTTTCTAGAGACGCCGCAATGATTAACAGGAACAGTCGGGGGCATCAGTATTCAGTTGTCAGAGG
TGAAATTCTTGGATTTACTGAAGACTAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAGTGAACG
AAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCGTAAACTATGCCGACTAGGGATCGGGCGA
TGTTCTTTTTCTGACTCGCTCGGCACCTTACGAGAAATCAAAGTTTTTGGGTTCTGGGGGGATTATGGTCGCAAG
GCTGAAACTTAAAGAAATTGACGGAAGGTCACCACCAGGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGG
AAACTCACCAGGTCCAGATGAAATAAGGATTGACAGATTGAGAGCTCTTTCTTGATTTTTCAGGTGGTGGTGCAT
GGCCGTTCTTAGTTCGTGGGGTGACTTGTCTGCTTAATTGCGATAACGAGCGAGACCTTACTCTGCTAAATAGCC
AGGCTAACTTTGGTTGGTCGCCGGCTTCTTAGAGAGACTATCAACTCAAGTTGATGGA
```

Fig. 2. Consensus sequence of *Alternaria japonica* (WHK-12)

Table 1. Fifteen sequences with maximum identity score from BLAST report

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
KF962959.1	*Alternaria alternata* strain HA4087	1493	1493	100%	0.0	99%
JN088533.1	*Alternaria alternata* strain FC007	1493	1493	100%	0.0	99%
HM165489.1	*Alternaria alternata* strain S-f6	1493	1493	100%	0.0	99%
GQ354822.1	*Alternaria japonica* strain HDJZ-ZWM-06	1493	1493	100%	0.0	99%
GU456294.1	*Alternaria maritima* strain CBS 126.60	1489	1489	100%	0.0	99%
DQ678031.1	*Alternaria alternata* isolate AFTOL-ID 1610	1489	1489	100%	0.0	99%
AF229504.1	*Alternaria alternata* ATCC 28329	1487	1487	100%	0.0	99%
U05194.1	*Alternaria alternata* AA6	1487	1487	100%	0.0	99%
HM216191.1	*Alternaria alternata* strain SRC1lrK2f	1483	1483	100%	0.0	99%
AF548106.1	*Ulocladium botrytis* strain UPSC 3539	1482	1482	100%	0.0	99%
DQ247812.1	*Pleospora herbarum* strain CBS 191.86	1476	1476	100%	0.0	99%
AF229513.1	*Pleospora herbarum* ATCC 11681	1476	1476	100%	0.0	99%
AF229508.1	*Alternaria cheiranthi* EGS 41-188	1476	1476	100%	0.0	99%
U43458.1	*Pleospora herbarum* NS3/NS4 region	1476	1476	100%	0.0	99%
U05201.1	*Pleospora herbarum* DAOM 150679	1476	1476	100%	0.0	99%

Table 2. Distance matrix of the 15 sequences with maximum identity score from BLAST report

WHK-12	1		0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.002
KF962959.1	2	0.000		0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.002
JN088533.1	3	0.000	0.000		0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.002
HM165489.1	4	0.000	0.000	0.000		0.000	0.000	0.000	0.001	0.001	0.000	0.002
GQ354822.1	5	0.000	0.000	0.000	0.000		0.000	0.000	0.001	0.001	0.000	0.002
GU456294.1	6	0.000	0.000	0.000	0.000	0.000		0.000	0.001	0.001	0.000	0.002
DQ678031.1	7	0.000	0.000	0.000	0.000	0.000	0.000		0.001	0.001	0.000	0.002
AF229504.1	8	0.001	0.001	0.001	0.001	0.001	0.001	0.001		0.000	0.001	0.002
U05194.1	9	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000		0.001	0.002
HM216191.1	10	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001		0.002
AF548106.1	11	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.004	0.004	0.002	

Host range of the spore suspension

The present investigation indicates that the indigenous pathogen, A. *japonica* have significantly narrow host range (Table 3). *E. crassipes* and *Chenopodium album* L. (Chenopodiaceae) were the only compatible host plant of *A. japonica* as observed in these studies. None of the other plants were found susceptible to the fungal inoculum. *A. japonica* appears to be a promising biological control agent of water hyacinth. The spore suspension of *A. japonica* also caused appreciable disease on *C. album*. But this does not designate *A. japonica* as a threat as the susceptible plant itself is a noted weed and an ecological difference persists between the aquatic and the land weeds. *A. japonica* has been known to infest Brassicaceae plants including cole crops (Mamgain *et al.* 2013) and cause pod spot of radish (Scott *et al*. 2012). But during the present study none of the plants from Brassicaceae were affected by the pathogen. Thus mycoherbicidal management of water hyacinth seems to have a bright future using the indigenous culture of *A. japonica.*

Host range of the culture filtrate: The culture filtrate of *A. japonica* caused phytotoxic damage to 9 out of 48 plant species tested, *viz*. *Trianthema portulacastrum* L., *Amaranthus viridis* L., *Sinapis alba* L., *Chenopodium album* L., *Spinacia oleracea* L., *Ipomoea aquatic* Forsk, *Hydrilla verticillata* (L. f.) Royle, *Rumex obtusifolia* L., other than *E. crassipes*. Phytotoxic symptoms were produced on several of the plants tested, demonstrating that phytotoxin produced by *A. japonica* is although effective but not host specific. However looking into its damage potential against water hyacinth further intensive studies including proper knowledge of the toxic compounds produced by the fungus is essential. Further using biotechnological approaches (Miller *et al*. 1987) efforts can be directed towards limiting its host range.

Table 3. Host range testing of *A. japonica* on various crops and weed hosts

S.no.	Family	Common name	Vernacular name (in India)	Botanical name	Spore suspension (5×10^5 spores/ml)	Culture filtrate
1.	Aizoaceae	Horse-purslane	Pathar chata	*Trianthema portulacastrum* L.[c]	-	+
2.	Amaranthaceae	Alligator weed	Pani-khutura	*Alternanthera philoxeroides* (Mart.) Griseb. [b c]	-	-
3.		Sessile joyweed	Kantewali santhi	*Alternanthera sessilis* L. [c]	-	-
4.		Amaranth	Chaulai	*Amaranthus viridis* L. [a]	-	+
5.	Apiaceae	Asian pennywort	Brahmi	*Centella asiatica* L. [c]	-	-
6.	Araceae	Water lettuce	-	*Pistia stratiotes* L. [b c]	-	-
7.	Asteraceae	False oxtongue	Kukurbanda	*Blumea lacera* DC [c]	-	-
8.		Chickory	Kasani	*Cichorium intybus* L. [c]	-	-
9.		Parthenium	Gajar ghas	*Parthenium hysterophorus* L. [c]	-	-
10.		Perennial sowthistle	Bhatkataiya	*Sonchus arvensis* L. [c]	-	-
11.		Marigold	Genda	*Tagetes erecta* L. [a]	-	-
12.		Coat buttons	Phulani	*Tridax procumbens* L. [c]	-	-
13.	Brassicaceae[d]	Rai	Sarson	*Brassica campestris* L. *var sarson* [a]	-	-
14.		Radish	Mooli	*Raphanus sativus* L.	-	-
15.		Cauliflower	Phool gobhi	*B. oleracea* L. *var. botrytis* [a]	-	-
16.		Cabbage	Bandha gobhi	*B. oleracea* L. *var. capitata* [a]	-	-
17.		Wild mustard	Safed Rai	*Sinapis alba* L. [c]	-	+
18.	Chenopodiaceae	Goosefoot	Bathua	*Chenopodium album* L. [a c]	+	+
19.		Spinach	Palak	*Spinacia oleracea* L. [a]	-	+
20.	Commelinaceae	Tropical Spiderwort	Kanteri	*Commelina benghalensis* L. [c]	-	-
21.	Convolvulaceae	Bindweed	Hiran chara	*Convolvulus arvensis* L. [c]	-	-
22.		Morning glory	Beshram	*Ipomoea fistulosa* Mart. [c]	-	-
23.		Water spinach	Kalmi sag	*Ipomoea aquatic* Forsk[b c]	-	+
24.	Cyperaceae	Rice foot sedge	Galmotha	*Cyperus iria* L. [c]	-	-
25.	Euphorbiaceae	Asthma weed	Dudhi	*Euphorbia hirta* L. [c]	-	-
26.	Fabaceae					
27.		Gram	Chana	*Cicer arietinum* L. [a]	-	-
28.		Soybean	Soybean	*Glycine max* L. [a]	-	-
29.		Lentil	Masoor	*Lens esculenta* Moench [a]	-	-
30.		Medick	-	*Medicago polymorpha* L. [c]	-	-
31.		Pea	Matar	*Pisum sativum* L. [a]	-	-
32.		Egyptian clove	Barseem	*Trifolium alexandrium* L. [c]	-	-
33.		Mung bean	Moong	*Vigna radiata* L. [a]	-	-
34.	Gramineae	Para grass	-	*Brachiaria mutica* (Forsk.) Stapf. [c]	-	-
35.		Bermuda grass	Dubh	*Cynodon dactylon* L. [c]	-	-
36.		Paddy	Dhan	*Oryza sativa* L. [a]	-	-
37.		Wheat	Gehoon	*Triticum aestivum* L. [a]	-	-
38.		Maize, Corn	Bhutta, Makka	*Zea mays* L. [a]	-	-
39.	Hydrocharitaceae	Hydrilla	-	*Hydrilla verticillata* (L. f.) Royle [b c]	-	+
40.	Lamiaceae	Pignut	Wilayati tulsi	*Hyptis suaveolens* L. Point. [c]	-	-
41.	Lemnaceae	Common duckweed	-	*Lemna minor* L. [b c]	-	-
42.	Linaceae	Linseed	Alsi	*Linum usitatissimum* L. [a]	-	-
43.	Malvaceae	Common wire weed	Kareta	*Sida acuta* Burm. f. [c]	-	-
44.	Polygonaceae	Broad-leaved dock	Jungli palak	*Rumex obtusifolius* L. [c]	-	+
45.	Pontederiaceae	Water hyacinth	Jal kumbhi	*Eichhornia crassipes* (Mart.) Solms	+	+
46.	Solanaceae	Tomato	Tamaatar	*Lycopersicon esculentum* Mill. [a]	-	-
47.	*Verbenaceae*	Wild gooseberry	Pachkotta	*Physalis minima* L. [c]	-	-
		Lantana		*Lantana camara L.* [c]	-	-

[a] Cultivated plant, [b] Plant ecologically related to the test plant, [c] Weed, [d] Plant reported susceptible to cultivars of *A. japonica*, *Spore suspension was sprayed in water containing hydrilla while phytotoxicity was accessed by growing the hydrilla shoot in the culture filtrate., + damage caused, - no damage

ACKNOWLEDGEMENTS

The authors express their heartfelt gratitude to the research grant provided by the Science and Engineering Research Board, New Delhi (SERB Project No. SERB/F/5316/2013-14 dated 18.11.2013) and DBT-BUILDER Program, Presidency University, Kolkata (D.O. No.:BT/ PR11357/INF/22/197/2014, dated 28/03/2014) for undertaking this project.

REFERENCES

Bennett FD and Zwölfer H. 1968. Exploration for natural enemies of the water hyacinth in northern South America and Trinidad. *Hyacinth Control Journal* **7**: 44-52.

Center TD. 1994. Biological control of weeds: water hyacinth and waterlettuce. pp. 481-521. In: *Pest management in the Subtropics: Biological control – A Florida Perspectives.* (Eds.) Rosen D, Bennett FD and Capinera JL. Intercept Publ. Co., Andover, U.K.

Charudattan R. 2001. Biological control of water hyacinth by using pathogens: opportunities, challenges, and recent developments. pp.21–28. In: *Biological and integrated control of water hyacinth, Eichhornia crassipes. Proceedings of the Second Meeting of the Global Working Group for the Biological and Integrated Control of Water Hyacinth, Eichhornia crassipes* . (Eds.) Julien MH, Hill MP, Center TD, Jianqing D (eds), Beijing, China, 9–12 October, 2000. Australian Centre for International Agricultural Research (ACIAR) Proceedings No. 102.

Coetzee JA, Hill MP, Byrne MJ and Bownes A. 2011. A review of the biological control programmes on *Eichhornia crassipes* (C. Mart.) Solms (Pontederiacaeae), *Salvinia molesta* D.S.Mitch. (Salviniaceae), *Pistia stratiotes* L. (Araceae), *Myriophyllum aquaticum* (Vell.) Verdc. (Haloragaceae) and *Azolla filiculoides* Lam. (Azollaceae) in South Africa. *African Entomologist* **19**(2): 451-468.

Gopal B. 1987. *Water hyacinth. Aquatic plant studies* 1. Elsevier, Amsterdam- Oxford- New York – Tokyo.

Holm LG, Plucknett DL, Pancho JV and Herberger JP. 1977. *The World's Worst Weeds: Distribution and Biology.* Honolulu: University press of Hawaii.

Hong SG, Cramer RA, Lawrence CB and Pryor BM. 2005. Alt a 1 allergen homologs from *Alternaria* and related taxa: analysis of phylogenetic content and secondary structure. *Fungal Genetics and Biology* **42**: 119-129.

Julien MH and Orapa W. 2001. Insect used for biological control of aquatic weed water hyacinth in Papua New Guinea. *Journal of Agriculture, Forestry and Fisheries* **44** (1&2): 49-60.

Lawrence DP, Gannibal PB, Peever TL and Pryor BM. 2013. The sections of *Alternaria*: Formalizing species-groups concepts. *Mycologia* **105**: 530–546.

Mamgain A, Roychowdhury R and Tah J. 2013. *Alternaria* pathogenicity and its strategic controls. *Journal of Biological Research* **1**: 1-9.

Miller RV, Ford EJ and Sands DC. 1987. Reduced host-range mutants of S*clerotinia sclerotiorum. Phytopathology* **77**: 1695.

Nag Raj JR and Ponnappa KM. 1970. Blight of water hyacinth caused by *Alternaria alternata* sp. Nov. *Transactions of British Mycological Society* **55**: 123–130.

Pathak A and Kannan C. 2011. Isolation and pathogenicity of some native fungal pathogens for the biological management of water hyacinth. *Indian Journal of Weed Science* **44** (4): 178-180.

Pryor BM and Bigelow DM. 2003. Molecular characterization of *Embellisia* and *Nimbya* species and their relationship to *Alternaria*, *Ulocladium* and *Stemphylium*. *Mycologia* **95**: 1141-1154.

Pryor BM and Gilbertson RL. 2000. Molecular phylogenetic relationships amongst *Alternaria* species and related fungi based upon analysis of nuclear ITS and mt SSU rDNA sequences. *Mycological Research* **104**: 1312-1321.

Ray P and Hill MP. 2012. Fungi associated with *Eichhornia crassipes* in South Africa and their pathogenicity under controlled conditions. *African Journal of Aquatic Science* **37**(3): 323-331.

Ray P and Hill MP. 2013. Microbial agents for control of aquatic weeds and their role in integrated management. *CAB Reviews Perspectives in Agriculture Veterinary Science Nutrition and Natural Resources* **8**: 1-9.

Ray P, Sushilkumar and Pandey AK. 2008. Deleterious effect of herbicides on water hyacinth biocontrol agents *Neochetina bruchi* and *Alternaria alternata*. *Biocontrol Science and Technology* **18**(5): 523-533.

Ray P. 2006. *Management of water hyacinth employing some insects and fungi.* Ph D Thesis, R.D. University, Jabalpur (M.P.) India.

Scott J, Julien M, McFadyen R and Cullen J. 2012. *Raphanus raphanistrum* L.-wild radish. pp. 486-493. In: *Biological control of weeds in Australia*. (Eds.)Cullen J, Julien M, McFadyen R.

Shabana YM, Buka ZAM and Abdel-Fattah GM. (1997). *Alternaria eichhorniae*, a biological control agent of water hyacinth: mycoherbicidal formulation and physiological and ultrastructural host responses. *European Journal of Plant Pathology* **103**: 99-111.

Shabana YM. 2005. The use of oil emulsions for improving the efficacy of *Alternaria eichhorniae* as a mycoherbicide for water hyacinth. (*Eichhornia crassipes*). *Biological Control* **32**: 78–89.

Wapshere A. 1974. A strategy for evaluating the safety of organisms for biological weed control. *Annals of Applied Biology* **77**: 201-211.

Woudenberg JHC, Groenewald JZ, Binder M. and Crous PW. 2013. *Alternaria* redefined. *Studies in Mycology* **75:** 171-212.

Phytoparasitica (2017) 45:429–437
DOI 10.1007/s12600-017-0605-y

A glimpse into the compatibilities and conflicts between arthropods and fungal biological control agents of aquatic weed waterhyacinth

Writuparna Dutta · Puja Ray

Received: 16 February 2017 / Accepted: 23 July 2017 / Published online: 4 August 2017

Abstract Interspecific interactions play an important role in ecosystem functioning. Several biocontrol agents are being introduced for the control of invasive species like the aquatic macrophyte, waterhyacinth, *Eichhornia crassipes*, in the hope of improving the eco-friendly natural control mechanism. Due to their overlapping niches, interspecific interactions among the biocontrol agents is high and there is a paucity of information on possible positive or negative impact they may have on each other hence affecting the biocontrol. Therefore studies on their interaction become even more important. In order to understand such possibilities, three potential fungi, *Fusarium oxysporum* (FO), *F. roseum* (FR), *Paradendryphiella salina* (PS) and two host-specific arthropods *Orthogalumna terebrantis* (OT) and *Neochetina bruchi* (NB) were applied on water hyacinth plants singly and in various combinations (OT, NB, FO, FO + OT, FO + NB, FR, FR + OT, FR + NB, PS, PS + OT, PS + NB). Observations were made on percentage damage caused by these agents to water hyacinth. Biocontrol agents applied in combination had cumulative impact on the target weed. FO + NB and FO + OT treated plants showed enhanced damage percentage of about 90%, after 120 h. But FR + NB and PS + NB treated plants were far less damaged compared to when these agents were applied separately. PS also had a repelling effect on NB. Results of such interactions are often variable and less predictable, especially under field conditions. These results demonstrate the need for studies on biotic interactions among biocontrol agents, prior to release of the biocontrol agents of weeds.

Keywords Multitropic-interaction · Invasive species · Phytopathogen · Repulsive effect; weed biocontrol

W. Dutta
Multitrophic Interactions and Biocontrol Laboratory, Department of Life Sciences, Presidency University, 86/1 College Street, Kolkata -700073, India
e-mail: puja.ray@gmail.com

Present Address:
P. Ray (✉)

Multitrophic Interactions and Biocontrol Laboratory, Department of Life Sciences, Presidency University, 86/1 College Street, Kolkata -700073, India
e-mail: puja.ray@gmail.com

Introduction

Waterhyacinth, *Eichhornia crassipes* Mart. Solms-Laubach (Pontederiaceae), a native of the tropical South America, is considered to be one of the most serious aquatic weeds (Holm et al. 1977). The climatic conditions of tropical and sub-tropical countries have favoured the invasiveness of the weed, causing widespread problems to millions of users of aquatic bodies and its resources (Gopal 1987). Waterhyacinth invasiveness has led to a tremendous negative impact on the social and economic conditions of the aquatic ecosystem at various trophic-levels (Villamagna and Murphy 2010), causing a global annual loss of more than US$ 100 million to hydroelectricity generation, irrigation

ANNEXURE III:

Awards & Achievements

1. IWSC TRAVEL AWARD WINNER

Graduate Student Award and travel grant at *International Weed Science Congress* held at Prague, Czech Republic

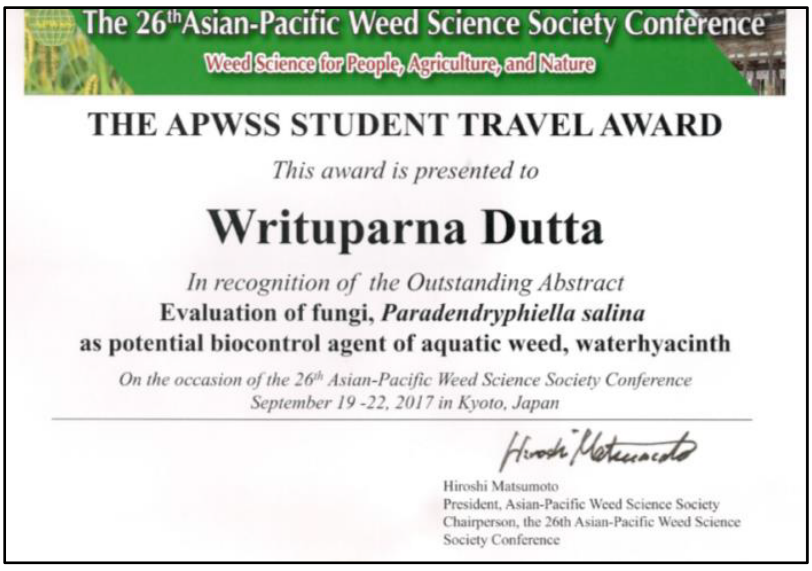
Title of Paper: Arthropod-Fungi Interaction in Biocontrol of *Eichhornia crassipes*

2. APWSS 2017 KYOTO STUDENT TRAVEL GRANT

The 26th APWSS Student Travel Grant with an amount of 30,000 JPY was awarded based on the best paper selected.

Poster: Evaluation of fungi, *P. salina* as a potential biocontrol agent of aquatic weed, water hyacinth

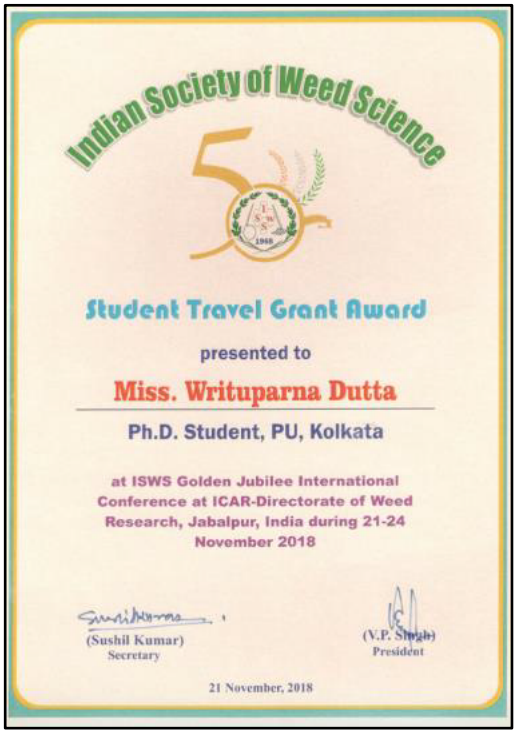
Oral: Studies on understanding the trends of invasive plant succession through inter-specific interactions among weeds



3. ISWS STUDENT TRAVEL FUND

The Student Travel Grant Fund Awardee for ISWS Biennial Conference, held at ISWS, International Golden Jubilee Conference,

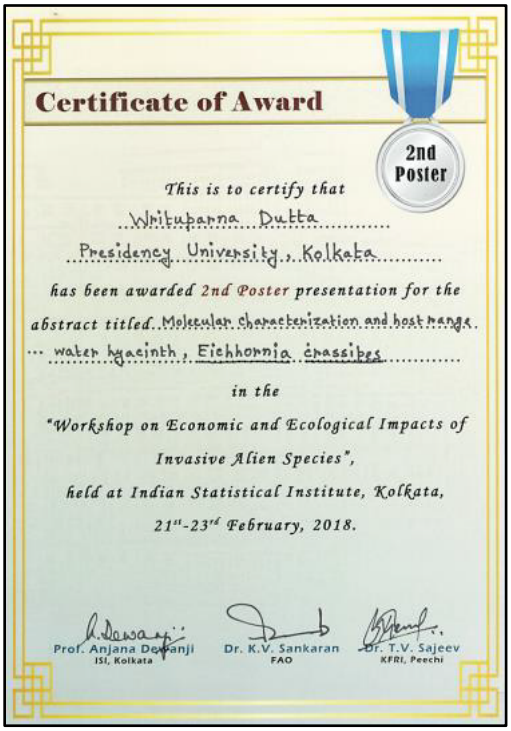
Oral: Impact of biocontrol agents on biochemical changes of aquatic weed water hyacinth, *Eichhornia crassipes* (Mart.) Solms-Laubach



4. 2ND PRIZE IN POSTER PRESENTATION

The 2nd Prize at Workshop on Economic and Ecological Impacts of Invasive Alien Species held at Indian Statistical Institute, Kolkata, India (21st – 23rd February 2018)

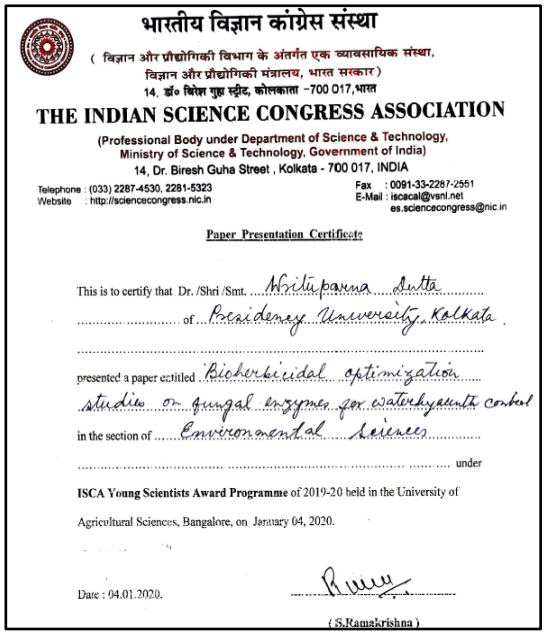
Poster: Molecular characterization and host range studies of *Alternaria japonica* Yoshii as a prospective mycoherbicidal agent of waterhyacinth, *Eichhornia crassipes*



5. ISCA 2020 YOUNG SCIENTIST FINALIST

The only finalist for the Young Scientist Award in Environmental Sciences Category of 107th Indian Science Congress held at Bangalore, India (3rd – 27th January 2020)

Oral: Bioherbicidal optimization studies on fungal enzymes for waterhyacinth control



भारतीय विज्ञान कांग्रेस संस्था

(विज्ञान और प्रौद्योगिकी विभाग के अंतर्गत एक व्यावसायिक संस्था,
विज्ञान और प्रौद्योगिकी मंत्रालय, भारत सरकार)
14, डॉ० बिरेश गुह स्ट्रीट, कोलकाता -700 017, भारत

THE INDIAN SCIENCE CONGRESS ASSOCIATION

(Professional Body under Department of Science & Technology,
Ministry of Science & Technology, Government of India)
14, Dr. Biresh Guha Street , Kolkata - 700 017, INDIA

Telephone : (033) 2287-4530, 2281-5323 Fax : 0091-33-2287-2551
Website : http://sciencecongress.nic.in E-Mail : iscacal@vsnl.net
es.sciencecongress@nic.in

Paper Presentation Certificate

This is to certify that Dr. /Shri /Smt. Writuparna Dutta
of Presidency University, Kolkata
presented a paper entitled Bioherbicidal optimization
studies on fungal enzymes for waterhyacinth control
in the section of Environmental Sciences under
ISCA Young Scientists Award Programme of 2019-20 held in the University of
Agricultural Sciences, Bangalore, on January 04, 2020.

Date : 04.01.2020.

(S.Ramakrishna)

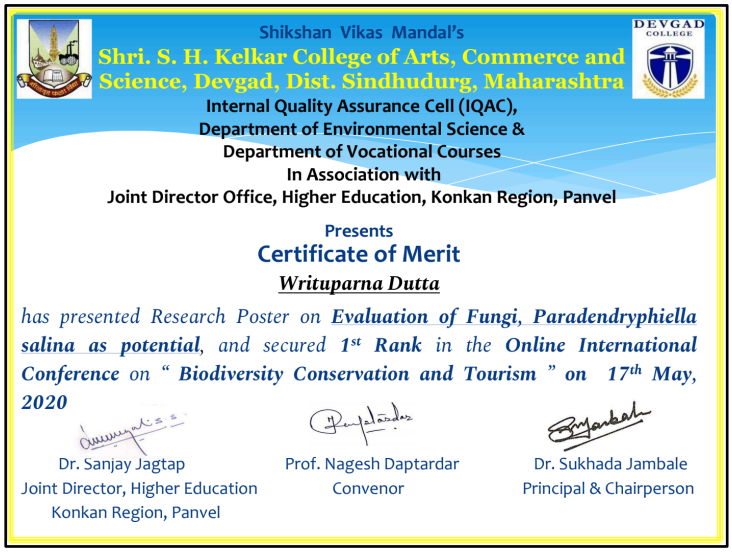
6. INTERNATIONAL ONLINE POSTER COMPETITION

The 1st Prize on Online International Conference on "BIODIVERSITY CONSERVATION AND TOURISM", under the theme Biodiversity and Environmental Sustainability

Organized by: Devgad College, Maharashtra, India

Scheduled on 17th May 2020

Poster: Evaluation of fungi, *P. salina* as a potential biocontrol agent of aquatic weed, water hyacinth



Shikshan Vikas Mandal's
Shri. S. H. Kelkar College of Arts, Commerce and Science, Devgad, Dist. Sindhudurg, Maharashtra
Internal Quality Assurance Cell (IQAC),
Department of Environmental Science &
Department of Vocational Courses
In Association with
Joint Director Office, Higher Education, Konkan Region, Panvel

Presents

Certificate of Merit

Writuparna Dutta

*has presented Research Poster on **Evaluation of Fungi, Paradendryphiella salina as potential**, and secured **1st Rank** in the **Online International Conference** on " **Biodiversity Conservation and Tourism** " on **17th May, 2020***

Dr. Sanjay Jagtap
Joint Director, Higher Education
Konkan Region, Panvel

Prof. Nagesh Daptardar
Convenor

Dr. Sukhada Jambale
Principal & Chairperson

ANNEXURE IV:

List of conferences and seminars presented with certificates

International Presentations:

1. **Dutta W**. and Ray P. (2020) Evaluation of fungi, *Paradendryphiella salina*, as a potential biocontrol agent of aquatic weed, water hyacinth. Online International Conference on “Biodiversity conservation and tourism", under the theme ‘Biodiversity and Environmental Sustainability’, Organized by: Devgad College, Maharastra, India. 17th May 2020. [Poster]
2. **Dutta W**., Misra D. and Ray P. (2018) Impact of biocontrol agents on biochemical changes of aquatic weed water hyacinth, *Eichhornia crassipes* (Mart.) Solms-Laubach. 50thISWS Golden Jubilee International Conference "Weed and Society: Challenges and Opportunities", ICAR-Directorate of Weed Research, Jabalpur, India. 21st-24th November 2018. [Oral]
3. **Dutta W**., Ray D. and Ray P. (2018) Molecular characterization and host range studies of a native phytopathogen as a prospective mycoherbicidal agent of water hyacinth, *Eichhornia crassipes*. Workshop on Economic and Ecological Impacts of Invasive Alien Species. Indian Statistical Institute, Kolkata, India. 21st-23rd February 2018. [Poster]
4. **Dutta W**. and Ray P. (2017) Evaluation of fungi, *Paradendryphiella salina*, as a potential biocontrol agent of aquatic weed, water hyacinth. 26thAsia-Pacific Weed Science Society Conference, Kyoto, Japan. 19th – 22nd September 2017. [Poster]
5. **Dutta W**., Chaudhury P. and Ray P. (2017) Studies on understanding the trends of invasive aquatic plant succession through inter-specific interactions among weeds. 26thAsia-Pacific Weed Science Society Conference, Kyoto, Japan. 19th–22nd September 2017. [Oral]
6. **Dutta W.**, Misra D., Ray D. and Ray P. (2017) Molecular characterization and host range studies of *a native phytopathogen* as prospective mycoherbicidal agent of waterhyacinth, *Eichhornia crassipes*. International Symposium on “Eco – efficiency in agriculture & allied research (EEAAR 17)”, Crop and Weed Science Society (CWSS), Bidhan Chandra Krishi Viswavidyalaya (BCKV), 20th-23rd January, 2017. [Poster]

7. **Dutta W**. and Ray P. (2016) Arthropod-Fungi Interaction in Biocontrol of *Eichhornia crassipes*. 7thInternational Weed Science Congress, Czech Republic, Prague. 19th – 25th June 2016. [Oral]
8. **Dutta W**., Ray P., Biswas Q., Ukil B., Halder S. and Majumder A. (2014). Diversity and abundance of various phytopathogens of *Echhornia crassipes* in Kolkata, India. International Conference on Environmental Biology and Ecological Modelling (ICEBEM)", Vishva-Bharati, Santiniketan, W.B. 24th-26th Feb 2014. [Poster]
9. **Dutta W**. and Ray P. (2014) An Assessment of Impact of Climate Change on Invasive Weeds. National Symposium entitled "*Modern Trends in Biological Sciences*", Physiological Institute, Presidency University, Kolkata. 21st February 2014. [Poster]

National Presentations:

1. **Dutta W.** (2020) Bioherbicidal optimization studies on fungal enzymes for water hyacinth control. Young Scientist Award Program. 107thIndian Science Congress Association (Section: Environmental Science). 3rd-7th January 2020. [Oral]
2. **Dutta W.**, Ganesan M. and Ray P. (2020) Fungal xylanases mediated biocontrol of invasive aquatic macrophyte water hyacinth: A new approach in weed management. National Conference on Plant Genomics. SRM IST, Tamil Nadu. 23rd-24th January 2020 [Poster]
3. **Dutta W**. and Ray P. (2017) Evaluation of fungi, *Paradendryphiella salina*, as a potential biocontrol agent of aquatic weed, water hyacinth. National Conference on Status of Invasive Alien Species in India, organized by Government of India; Ministry of Environment, Forest and Climate Change; Zoological Survey of India and Botanical Survey of India. Kolkata.14th-15th December 2017 [Poster]
4. **Dutta W**. and Ray P. (2016) Optimisation of enzymes from phytopathogenic fungi *Alternaria alternata* and their attempt in biological control. 23rd West Bengal State Science and Technology Congress, 2016, Affiliation: Department of West Bengal. 28th – 29th February 2016. [Oral]

5. Chaudhury P., Ray R., **Dutta W**. and Ray P. (2016) Impact of abiotic parameters on invasive weeds in respect to their interspecific-interactions. 23rd West Bengal State Science and Technology Congress, 2016, Affiliation: Department of West Bengal. 28th – 29th February 2016. [Oral]
6. Mal D., Seal S., Santra P., **Dutta W**. and Ray P. (2016) Non-native weeds are not always bad, their ecological role in supporting butterflies. 23 rdWest Bengal State Science and Technology Congress, 2016, Affiliation: Department of West Bengal. 28th – 29th February 2016. [Oral]
7. Ghosh S., **Dutta W**., Nandy S., Mal D., Ray R., Chaudhury P., Seal S., Santra P. and Ray P. (2016) Fungal metabolites as eco-friendly alternate to hazardous chemicals pesticides. 23 rdWest Bengal State Science and Technology Congress, 2016, Affiliation: Department of West Bengal. 28th – 29th February 2016. [Poster]
8. Sarkar S., **Dutta W**. and Ray P. (2015) Mycotoxins for biochemical control of aquatic weed, *Eichhornia crassipes* Poster presentation in a DBT-BUILDER sponsored National Symposium on "Environmental Impact on Biodiversity and Plant Development" at Presidency University, Kolkata on 19th – 20th February 2015. [Poster]
9. Mal D., **Dutta W**. and Ray P. (2015) Studies on Phytopathogens of Water Hyacinth for their biocontrol potential. Poster presentation in a DBT-BUILDER sponsored National Symposium on "Environmental Impact on Biodiversity and Plant Development" at Presidency University, Kolkata on 19th – 20th February 2015. [Poster]
10. Routh D., Mandal K., **Dutta W**. and Ray P. (2015) Human Mediated Drivers of Change-Impact on Butterflies. Poster presentation in a DBT-BUILDER sponsored National Symposium on "Environmental Impact on Biodiversity and Plant Development" at Presidency University, Kolkata on 19th – 20th February 2015. [Poster]
11. **Dutta W.**, Nandi S., Sarkar S, Mandal K., Routh D., Mal D. and Ray P. (2015) A glimpse into the world of fungal metabolites as eco-friendly alternate to hazardous chemicals. Symposium Molecules to Systems. Presidency University, Kolkata, 29th-31st Jan 2015. [Poster]
12. **Dutta W.** and Ray P. (2014) Attempts on enhancing enzymatic activities of phytopathogenic fungi utilizing noxious weed, water hyacinth. Symposium on

Recent Advances in Biotechnology for Food and Fuel. New Delhi. 19th - 20th November 2014. [Poster]

13. **Dutta W.** and Ray P. (2014) Attempts on using fungal enzymes in weed biocontrol. Symposium on Recent Advances in Biotechnology for Food and Fuel at New Delhi on 19th - 20th November 2014. [Oral]

CERTIFICATE OF CONFERENCES ATTENDED (MOSTLY PRESENTED ONES)

i. 2014

INTERNATIONAL CONFERENCE ON ENVIRONMENTAL BIOLOGY
AND
ECOLOGICAL MODELLING (ICEBEM 2014)

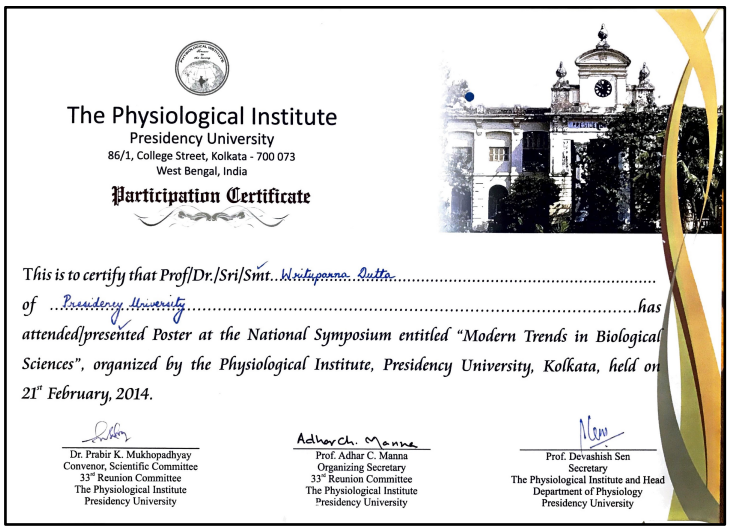
Department of Zoology
(Centre for Advanced Studies)
VISVA-BHARATI, SANTINIKETAN 731235

Certificate

This is to certify that Prof. / Dr. / Mr. / Mrs. / Miss / Srituparna Dutta participated / presented poster / delivered oral presentation / invited lecture / special lecture / acted as chairperson in the International Conference on of Environmental Biology and Ecological Modelling (ICEBEM 2014) organized by Department of Zoology, Visva-Bharati during 24 - 26 February, 2014.

Prof. V. C. Joy
Jt. Convener

Prof. Santanu Ray
Jt. Convener



The Physiological Institute
Presidency University
86/1, College Street, Kolkata - 700 073
West Bengal, India

Participation Certificate

This is to certify that Prof/Dr./Sri/Smt. Srituparna Dutta
of Presidency University has attended/presented Poster at the National Symposium entitled "Modern Trends in Biological Sciences", organized by the Physiological Institute, Presidency University, Kolkata, held on 21st February, 2014.

Dr. Prabir K. Mukhopadhyay
Convenor, Scientific Committee
33rd Reunion Committee
The Physiological Institute
Presidency University

Prof. Adhar C. Manna
Organizing Secretary
33rd Reunion Committee
The Physiological Institute
Presidency University

Prof. Devashish Sen
Secretary
The Physiological Institute and Head
Department of Physiology
Presidency University

ii. 2015

CERTIFICATE OF ATTENDANCE

DBT-BUILDER Sponsored National Symposium, February 19-20, 2015

"Environmental Impact on Biodiversity and Plant Development"

Certifies that

WRITUPARNA DUTTA

PRESIDENCY UNIVERSITY

has attended the National Symposium organized by the Department of Biological Sciences and presented an invited talk/poster

PRESIDENCY UNIVERSITY KOLKATA

Malay Das

Dr. Malay Das
Convener & Organizing Secretary

Dr. Madhubrata Choudhury
Head of the Department

Dr. Kamala Gupta
Treasurer

iii. 2016

23rd
WEST BENGAL STATE SCIENCE & TECHNOLOGY CONGRESS 2016
28–29 FEBRUARY, 2016

This is to certify that Prof/Dr/Mr/Mrs Miss Writuparna Dutta

of Presidency University, Kolkata participated/presented paper/delivered

invited talk/delivered lead lecture in Section Biochemistry and Biotechnology

and/or exhibited model at the **23rd West Bengal State Science and Technology Congress, 2016**

held during 28-29 February, 2016.

Title of the paper/model/exhibit Optimisation of enzymes from phytopathogenic fungi *Alternaria alternata* and their attempt in biological control

Upal Chakrabarti
Joint Organising Secretary

Soumendu Chatterjee
Joint Organising Secretary

7th International
Weed Science Congress
June 19–25, 2016
Prague, Czech Republic

Certificate of Attendance

We hereby certify that

Dutta Writuparna

has attended the

7th International Weed Science Congress

"Weed Science and Management to Feed the Planet"

held at the Clarion Congress hotel Prague, Czech Republic
June 19–25, 2016.

Josef Soukup
Chairman of the Local Organizing Committee

Bernal Valverde
Chairman of the Scientific Programme Committee

iv. 2017

Crop and Weed Science Society (CWSS),
Bidhan Chandra Krishi Viswavidyalaya (BCKV)
on the occasion of International Symposium on "Eco – Efficiency in Agriculture & Allied Research"
at Farmers' Academy & Convention Centre (FACC), BCKV, Kalyani,Nadia, West Bengal, India
January 20-23,2017

CROP AND WEED SCIENCE SOCIETY • ESTD. 2004 •

Certificate

Prof. / Dr. / Mr. / Mrs. / Ms. Writuparna Dutta

In acknowledgment to your Presentation of Scientific article (Invited paper / Oral presentation / Poster presentation / Participation in Scientific Programme of International Symposium of "Eco – Efficiency in Agriculture & Allied Research"

Prof. R.K.Ghosh
Working President CWSS and
Organizing Secretary INS EEAAR 17

Prof. P.K.Sahu
Secretary, CWSS and
Joint Secretary INS EEAAR 17

Prof. A. Bhattacharyya
President CWSS and
President INS EEAAR 17

CROP AND WEED SCIENCE SOCIETY (CWSS)
Secretariat: Department of Agronomy, Faculty of Agriculture,
Bidhan Chandra Krishi Viswavidyalaya (BCKV), Mohanpur - 741252, West Bengal, India
Website: http://cwssbckv.org / www.cropandweed.com

p57

The 26th Asian-Pacific Weed Science Society Conference

APWSS 2017 Kyoto, JAPAN

The Golden Jubilee Conference

Weed Science for People, Agriculture, and Nature

Celebrating 50 Years!

Certificate of Presentation

Writuparna Dutta

presented "**Evaluation of fungi, *Paradendryphiella salina* as potential biocontrol agent of aquatic weed, waterhyacinth**" as a speaker in the 26th Asian-Pacific Weed Science Society Conference (APWSS2017) , held between September 19th-22nd, 2017 in Kyoto, Japan.

Hiroshi Matsumoto
Chairperson, the 26th Asian-Pacific Weed Science Society Conference (APWSS2017)
President, Asian-Pacific Weed Science Society
Professor, University of Tsukuba

The 26th Asian-Pacific Weed Science Society Conference

APWSS 2017 Kyoto, JAPAN

The Golden Jubilee Conference

Weed Science for People, Agriculture, and Nature

Celebrating 50 Years!

Certificate of Presentation

Writuparna Dutta

presented "**Studies on understanding the trends of invasive aquatic plant succession through inter-specific interactions among weeds**" as a speaker in the 26th Asian-Pacific Weed Science Society Conference (APWSS2017) , held between September 19th-22nd, 2017 in Kyoto, Japan.

Hiroshi Matsumoto
Chairperson, the 26th Asian-Pacific Weed Science Society Conference (APWSS2017)
President, Asian-Pacific Weed Science Society
Professor, University of Tsukuba

भारत सरकार / Government of India

पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय / Ministry of Environment, Forest and Climate Change

भारतीय प्राणि सर्वेक्षण / Zoological Survey of India

और / and

भारतीय वनस्पति सर्वेक्षण / Botanical Survey of India

Certificate

This is to certify that **Ms. Writuparna Dutta, Presidency University, Kolkata** *participated and made a poster presentation in the 'National Conference on Status of Invasive Alien Species in India' organized by Zoological Survey of India and Botanical Survey of India on 14th and 15th December, 2017 at Kolkata.*

Dr. Kailash Chandra
Director
Zoological Survey of India

Dr. Paramjit Singh
Director
Botanical Survey of India

v. 2018

Certificate
OF PARTICIPATION

APFISN Asia-Pacific Forest Invasive Species Network

This is to certify that **Ms. Writuparna Dutta,** Presidency University, Kolkata, has participated and made a poster presentation entitled "Molecular characterization and host range studies of Alternaria japonica Yoshii as prospective mycoherbicidal agent of water hyacinth, Eichhornia crassipes" in the "Workshop on Economic and Ecological Impacts of Invasive Alien Species", held at Indian Statistical Institute, Kolkata, 21st - 23rd February, 2018.

Prof. Anjana Dewanji
ISI, Kolkata

Dr. K.V. Sankaran
FAO

Dr. T.V. Sajeev
KFRI, Peechi

ISWS Golden Jubilee International Conference

"Weeds and Society: Challenges and Opportunities"

Certificate of Participation

This is to certify that Miss. Writuparna Dutta

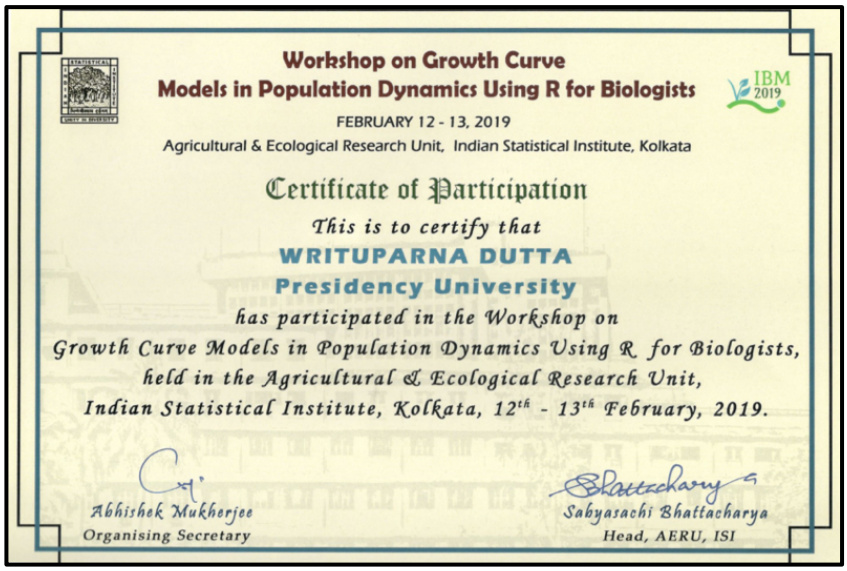
from Presidency University, Kolkata, West Bengal

participated and made an oral presentation in the ISWS Golden Jubilee International Conference held at ICAR-Directorate of Weed Research in Jabalpur, India during 21-24 November, 2018.

(Sushil Kumar)
Organizing Secretary

(V. Pratap Singh)
President, ISWS

vi. 2019



Workshop on Growth Curve Models in Population Dynamics Using R for Biologists

IBM 2019

FEBRUARY 12 - 13, 2019

Agricultural & Ecological Research Unit, Indian Statistical Institute, Kolkata

Certificate of Participation

This is to certify that

WRITUPARNA DUTTA

Presidency University

has participated in the Workshop on Growth Curve Models in Population Dynamics Using R for Biologists, held in the Agricultural & Ecological Research Unit, Indian Statistical Institute, Kolkata, 12th - 13th February, 2019.

Abhishek Mukherjee
Organising Secretary

Sabyasachi Bhattacharya
Head, AERU, ISI

vii. 2020

THE INDIAN SCIENCE CONGRESS ASSOCIATION

(Professional Body under Department of Science & Technology, Ministry of Science & Technology, Government of India)

14, Dr. Biresh Guha Street, Kolkata – 700 017

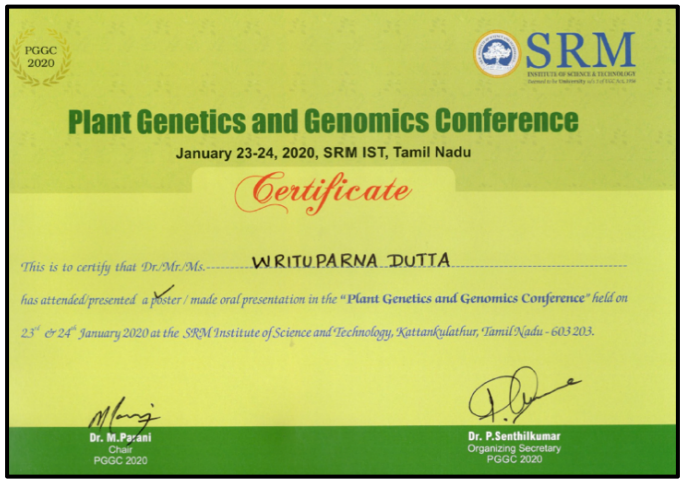
Participation Certificate

This is to certify that Prof./Dr./Shri/Smt. Writuparna Dutta of Presidency University, Kolkata has participated in the 107th Indian Science Congress held at University of Agricultural Sciences, Bangalore from January 3 to 7, 2020.

His/Her Membership Number is L30745

Date 7th January, 2020

Dr. S. Ramakrishna, General Secretary (Membership Affairs) — Office Seal — Dr. Anoop Kumar Jain, General Secretary (Scientific Activities)



PGGC 2020

SRM

Plant Genetics and Genomics Conference

January 23-24, 2020, SRM IST, Tamil Nadu

Certificate

This is to certify that Dr./Mr./Ms. WRITUPARNA DUTTA has attended/presented a poster / made oral presentation in the "Plant Genetics and Genomics Conference" held on 23rd & 24th January 2020 at the SRM Institute of Science and Technology, Kattankulathur, Tamil Nadu - 603 203.

Dr. M.Parani, Chair, PGGC 2020 — Dr. P.Senthilkumar, Organizing Secretary, PGGC 2020

Shikshan Vikas Mandal's

Shri. S. H. Kelkar College of Arts, Commerce and Science, Devgad, Dist. Sindhudurg, Maharashtra

DEVGAD COLLEGE

Internal Quality Assurance Cell (IQAC),
Department of Environmental Science &
Department of Vocational Courses
In Association with
Joint Director Office, Higher Education, Konkan Region, Panvel

Presents

Certificate of Participation

Writuparna Dutta

of ***Presidency University, Kolkata****, as an expression of gratitude for participation in the Online International Conference on "****Biodiversity Conservation and Tourism" on 17th May, 2020****.*

Dr. Sanjay Jagtap, Joint Director, Higher Education, Konkan Region, Panvel — Prof. Nagesh. Daptardar, Convenor — Dr. Sukhada Jambale, Principal & Chairperson