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PHYSICAL AND CHEMICAL FACTORS AFFECTING BIOMASS AND ALPHA TOXIN PRODUCTION OF *CLOSTRIDIUM PERFRINGENS* TOXINOTYPE A

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ABSTRACT

Clostridium perfringens toxinotype A is one of the important causes of diarrhea/enterotoxaemia in small and large ruminants. Various vaccines have been used against it in Pakistan but all are imported. To muddle through frequent onset of disease present study was conducted to optimized the various physical and chemical factors for maximum production of biomass and alpha toxin of *C. perfringens* type A. Biochemically confirmed indigenous isolates (n=3) were characterized by 16S rRNA gene amplification, followed by sequencing. These sequences were submitted in GenBank by the following accession no: MW332257.1, MW551947.1 and MW471067.1. Toxinotype A was confirmed by amplification of alpha-toxin gene and sequenced. Biomass and alpha-toxin were optimized for various physical (pH, time, and temperature) and chemical (carbohydrates, vitamin, mineral mixture, tween 80, ammonium chloride, sodium and potassium salts) parameters. Higher biomass was produced by MW551947.1 at 37°C with 7.5 pH (60.58±1.11 mg/mL) of RCM broth and at 0.5% sodium chloride (34.51±0.50 mg/mL) concentration for optimization after 48 hours of incubation. Furthermore, higher hemolytic units of alpha-toxin were produced by MW551947.1 at 37°C (6.07±0.12 HU/mL) and 0.3% glucose (47.73±0.23HU/mL) concentration with 6.5pH of broth after 24 hours of incubation. Under these optimized parameters, maximum biomass and alpha-toxin may be produced for cost-effective vaccine production on commercial basis.

Key words: Alpha toxin, Biomass, Glucose, r RNA, Sodium chloride, Toxinotype A

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INTRODUCTION

Clostridium perfringens is a gram-positive, rod-shaped, non-motile, spore-forming pathogenic anaerobic bacteria of human and domestic animals (Khiav and Zahmatkesh, 2021). *C. perfringens* is divided into 5 types A, B, C, D and E based on major toxins (α, β, ε, ι) and minor toxins (*netB*, *cpb2* and *cpe*) (Rood *et al*., 2018). *C. perfringens* is distributed in the environment i.e., soil, feces, sewages, foods and in the intestine of healthy human beings and animals as normal flora and responsible for human and animal diseases; these include enteritis and enterotoxaemia etc (McClane *et al*., 2006). *C. perfringens* toxinotype A produces alpha-toxin while the production of additional virulence factors including various minor toxins is variable (Li *et al.,* 2013). Enterotoxaemia is a condition in which toxins produced by *C. perfringens* absorb in to circulation and damage the kidney, lungs, liver and brain cells (McClane *et al*., 2006). Toxinotype A is also invasive and causes histotoxic infection called gas gangrene (Cavalcanti *et al.,* 2004). In young ruminants, it also causes hemorrhagic abomasitis, often accompanied by diarrhea (Heller *et al.,* 2018). The enterotoxaemia outbreaks occur due to poor feeding management and improper vaccination of small ruminants. Antimicrobial drugs and vaccines are used to control enterotoxaemia. The extensive use of antimicrobial agents for the treatment of gastro-intestinal infection is also responsible for antibiotic resistance and transfer of resistance genes (Ahsanullah *et al.,* 2019). To control enterotoxaemia, proper isolation of indigenous toxinotype in a particular area for vaccine production is essential. Furthermore, the continued selection of toxigenic strains that produce high toxin titer is pre-requisite (Bratbak and Dundas*,* 1984).

The livestock industry is the fastest growing and financially important sector of Pakistan economy, accounting 60.07% value addition to the Pakistan agricultural sector and contributing 11.53% in gross domestic product (GDP) of Pakistan (Anonymous, 2021). To cope with the high economic impact of *C. perfringens* and prevention of associated diseases is a big challenge for farmers. The development and production of conventional clostridial vaccines involve expensive, time-

consuming and dangerous processes of antigen concentration, purification and detoxification steps (Nijland *et al.,* 2007). So, optimization of toxins is required to define the optimum physico-chemical conditions. The present study is based on the effect of different physico-chemical conditions on biomass and alpha-toxin production potential of *C. perfringens* toxinotype A.

MATERIALS AND METHODS

C. perfringens toxinotype A (n = 3) previously isolated from sheep and goats were procured from the Institute of Microbiology, project TDF02-028, UVAS, Lahore, Pakistan.

Revival and growth on the specific medium of bacteria: *C. perfringens* toxinotypes were revived from microbead stock. Bead stock inoculated in sterile fluid thioglycollate broth (FTG) (Alanazi *et al.,* 2018). FTG medium was supplemented with equal volume mixture of 4 % sodium sulfite and 7 % ferric citrate (0.5 mL/25 mL: mL= milli liter), along with polymixin B (30,000 IU/L: IU/L= International units per liter), kanamycin sulfate (12 mg/L: mg/L= milli gram per liter) and D-cycloserine (400 mg/L). Inoculated medium tubes were incubated at 37ºC for 24-48 hours under anaerobic conditions in an anaerobic jar (IndiaMART®) using an anaerobic sachet (OXOID®). Revived isolates were cultured on sterile perfringens agar base medium (Lab M) petri plates supplemented with components mentioned above. Microscopic morphology was identified by gram's staining and spore staining.

Molecular Confirmation: Deoxyribose Nucleic Acid (DNA) was extracted by DNA extraction kit (WizBio) according to the manufacturer's recommendations. Extracted DNA was visually confirmed by agarose gel electrophoresis using 0.8% agarose gel. **16S rRNA gene typing:** Isolates were confirmed by PCR using 16S rRNA gene-specific primers following the method of Asghar *et al.* (2016) and the primer sequences used were Forward: 8FLP: 5`-AGTTTGATCCTGGCTCAG-3`, Reverse: XB4: 5`-GTGTGTACAAGGCCCGGGAAC-3`. The reaction mixture was prepared by mixing 2µL (µL= micro liter) of template DNA with 12.5µL of 2X PCR master mix (BioShop) or 1µL of each forward and reverse primer (10pmole: pmole= pico mole). To make the PCR mixture up to 25µL DNAse and RNAse free water 8.5µL was added. PCR was carried out in thermocycler (Kyretac) at 94ºC for 10 minutes, 35 cycles were at 94°C/1minute, 55°C/1minute, 72°C/2minute, and a final extension 72ºC/10minutes.

Molecular Toxinotyping: For molecular toxinotyping, *C. perfringens* toxins gene-specific primers were used to target the gene amplification. Isolates were confirmed by PCR using alpha, beta, epsilon and iota gene-specific primers following the method of Van Asten *et al.* (2009) and the primer sequences are represented in Table 1. PCR master mix (2X) (BioShop) 12.5µL, 1µL of each primer forward or revere (10pmole), 8.5µL nuclease-free water mixed with 2µL of extracted DNA. The tube was placed in a thermo cycler for amplification by providing 94ºC for 10 minutes for initial denaturation. Thirty-five (35) cycles were executed at 94°C for 1 minute for denaturation, 53ºC for 45seconds for annealing and 30 seconds for the extension at 72°C. A step of final extension at 72°C for 10 minutes was included.

Table 1. *C. perfringens* toxin gene-specific primers.

Genes	Primers	Primer sequences	Amplicons size (bp)
Alpha (*cpa*)	CPALPHA-F	5′-GCTAATGTTACTGCCGTTGA-3′	324
	CPALPHA-R	5′-CCTCTGATACATCGTGTAAG-3′	
Beta (*cpb*)	CPBetaF3	5′-GCGAATATGCTGAATCATCTA-3′	197
	CPBetaR3	5′-GCAGGAACATTAGTATATCTTC-3′	
Epsilon (*etx*)	CPEpsilonF	5′-TGGGAACTTCGATACAAGCA-3′	376
	CPEpsilonR2	5′-AACTGCACTATAATTTCCTTTTCC-3′	
Iota (*iap*)	CPIotaF2	5′-AATGGTCCTTTAAATAATCC-3′	272
	CPIotaR	5′-TTAGCAAATGCACTCATATT-3′	

bp: base pair(s)

Gel electrophoresis, sequencing and phylogenetic analysis: Amplification was confirmed by agarose gel electrophoresis using 1.5 percent gel concentration having ethidium bromide (0.5µg/mL). Gel results were visualized and recorded as mentioned above. Ribosomal RNA gene amplicons were subjected to sequencing and the FASTA file was received (Matsuo *et al.,* 2021). These sequences were cleaned using JUSTbio software and submitted to NCBI through GenBank and accession numbers were received. Based on these sequences, phylogenetic analysis was performed using MEGA X software. Phylogenetic tree for *C. perfringens* was made using 16S rRNA sequences, neighbor-joining algorithm, bootstrap as phylogeny method and 2000 bootstrap

replications. Based on alpha-toxin gene sequences, phylogenetic analysis was also performed (Kumar *et al.,* 2019).

Biomass and Toxin optimization under physico-chemical conditions: Bacterial cell inoculum for optimization was prepared following the method of Fernandez-Miyakawa *et al.* (2007) in normal saline (0.85% NaCl) containing L - cysteine HCl (0.05 %). Bacterial fresh growth on perfringens agar base medium (Lab M) was used for this purpose. Under sterile conditions loop full culture of *C. perfringens* type A (fresh growth) was added in normal saline and vortexed for mixing. Inoculum 1 McFarland was prepared by adjusting the suspension optical density to 0.248 using a spectrophotometer (Stalwart®) at 630 nm. Under sterile conditions, sterile labeled tubes of reinforced clostridial medium (RCM) broth (with supplements) were inoculated with 1 McFarland suspension (10% v/v) of *C. perfringens* type A. Physical conditions for biomass and toxin production were optimized following the method of Rai *et al.* (2013). Inoculated RCM broths of pH 6.5, 7.0, 7.5 and 8.0 were incubated at 37, 40, 42°C for 24, 36 and 48 hours under anaerobic conditions. After every time interval sample was taken out under sterile conditions and checked for toxin production. Chemical conditions for biomass and toxin production were optimized following the method of Rai *et al.* (2013). Carbohydrates (glucose, sucrose, dextrin: 0.1, 0.2 and 0.3%), mineral mixture (Iron, Magnesium, Zinc and Copper: 0.1, 0.2 and 0.3%), vitamin mixture (vitamin B-complex and C: 0.1, 0.2 and 0.3%), nitrogen source (ammonium chloride NH_4Cl: 0.5, 0.75, 1 and 1.5%), surfactant (tween 80: 0.1, 0.3 and 0.5%), and inorganic salts (sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate and sodium acetate: 0.1, 0.3 and 0.5%) were supplemented in to sterile RCM broth. After inoculation, incubation was done under anaerobic conditions by using optimized physical conditions.

Biomass estimation: Biomass was estimated by weight through analytical balance following the method of Bratbak and Dundas (1984). After growth under physicochemical conditions culture was centrifuged in 15mL falcon tubes at 8000 rpm for 5 to 10 minutes. Cell free supernatant was decanted, cell pellets were air-dried and weighted on an analytical balance. Weight of pellet was calculated by subtracting the weight of the empty tube from the total weight of the tube and cell pellet.

Hemolytic activity assay: Cell free supernatant was obtained by centrifugation of broth cultures in 15 mL centrifuge tube at 8000 rpm for 5 minutes. Cell-free supernatant was dispensed into sterile screw capped test tubes and stored at 4°C. Alpha toxin hemolytic activity was determined by microtiter plate assay, following the method of Nasir *et al.* (2015) and Hu *et al.* (2016). Alpha toxin containing cell-free supernatant was serially diluted (two fold) v/v in normal saline (NaCl 0.85%, pH 7.5) using microtiter (96well) round bottom plate (SPL). One percent washed sheep red blood cells (RBCs) prepared in sterile phosphate buffer saline (PBS: NaCl 8 g/L, KCl 200 mg/L, Na_2HPO_4 1.44 g/L and KH_2PO_4 240 mg/L, pH 7.4). In each well 100 µL 1 % sheep RBCs were added. Plates were incubated for 1 hour at 37°C with shaking. Optical density was measured by a spectrophotometer at 595 nm. And 50% hemolytic units (HU/mL) were recorded.

RESULTS

Molecular confirmation and toxinotyping: The genome of *C. perfringens* isolates was targeted for 16S rRNA gene amplification and 1500 bps size bands were observed (Fig. 1A) and after sequencing these sequences were submitted to GenBank with the following accession numbers: MW332257.1, MW551947.1 and MW471067.1.

Phylogenetic analysis was performed using MEGA-X (Fig. 2). In the phylogenetic tree, indigenous *C. perfringens* sequence's accession numbers were represented as a colored star. MW556208.1 was 70% evolutionary related to MW551947.1 Pak *C. perfringens*. MW332257.1 was 49% evolutionary related to MW332258.1 while MW556208.1 and MW551947.1 Pak *C. perfringens* was 60% related to LC386311.1 and 28% related to MW471067.1 Pak *C. perfringens*. The molecular toxinotyping represented that isolates bear alpha-toxin gene only (324 amplicons) (Fig.1B). The amplicons were sequenced and phylogenetic analysis was performed using MEGA-X (Fig. 3). Phylogenetic analysis based on alpha-toxin gene sequences revealed that Pakistan *C. perfringens* type A alpha-toxin CPS56 and CPG209 were 41% evolutionary related to each other and 68% associated with MK180785.1 alpha-toxin. CPG160 was 100% evolutionary related to CPS56, CPG209 and MK180785.1 alpha-toxin. CPS56 was 59% associated with MK180788.1 and MK180796.1.

Biomass optimization: Biomass optimization for *C. perfringens* toxinotype A isolates was carried out under different physical and chemical parameters. Type A isolates has produced higher biomass at 37ºC (55.53±0.50, 55.32±0.56 and 54.63±1.10 mg/mL) after 48 hours of incubation. With the increase of temperature from 40 to 42ºC, decrease in biomass was observed. Under the effect of pH and time of incubation, type A isolate MW551947.1 has produced higher biomass at 7.5pH 60.58±01.11 mg/mL after 48 hours of the time of incubation (Table 2).

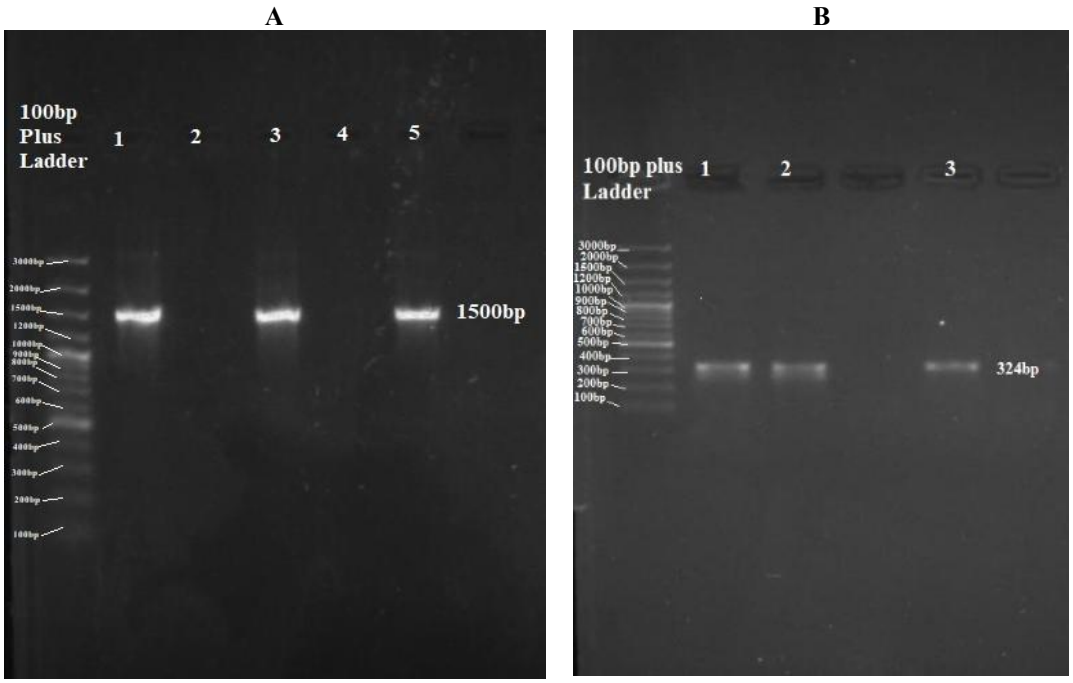

Fig. 1. PCR amplification of 16S rRNA and alpha-toxin gene of *C. perfringens* type A. A. Ribosomal 16S RNA gene amplification resulted in 1500bps amplicon visualized on 1.5% agarose gel. B. Alpha toxin gene amplification resulted in 324bps amplicon visualized on 1.5% agarose gel.

Table 2. *C. perfringens* Type A: Biomass optimization under physical parameters.

Temperature (ºC)	Time (Hours)	Biomass mg/mL		
		MW332257.1	**MW551947.1**	**MW471067.1**
	24	31.33±1.52 $^{a, b}$	30.37±0.56 ^{a}	33.27±0.51 ^{b}
37	36	41.36±1.51 ^{a}	41.11±0.06 ^{a}	44.34±1.28 ^{b}
	48	55.53±0.50 ^{a}	55.32±0.56 ^{a}	54.63±1.10 ^{a}
	24	9.00±0.20 $^{a, b}$	9.47±0.43 ^{b}	8.62±0.46 ^{a}
40	36	10.40±0.52 ^{a}	10.43±0.37 ^{a}	11.47±0.43 ^{b}
	48	9.96±1.00 ^{a}	9.92±0.06 ^{a}	9.28±0.41 ^{a}
	24	8.00±0.10 $^{a, b}$	7.92±0.04 ^{a}	8.10±0.01 ^{b}
42	36	7.70±0.10 ^{a}	7.64±0.087 ^{a}	7.79±0.005 ^{a}
	48	7.70±0.26 $^{a, b}$	7.574±0.10 ^{a}	8.02±0.04 ^{b}
pH				
	24	29.04±0.17^{a}	29.05±0.18^{a}	29.23±0.22^{a}
6.5	36	43.89±0.17^{a}	44.32±0.56^{a}	43.79±0.17^{a}
	48	59.32±0.76^{a}	59.61±0.60^{a}	58.88±0.10^{a}
	24	33.03±0.07^{a}	33.24±0.44^{a}	33.08±0.07^{a}
7.0	36	42.04±0.16^{a}	41.62±0.56^{a}	42.25±0.27^{a}
	48	56.00±0.00^{a}	56.32±0.56^{a}	56.18±0.31^{a}
	24	35.00±0.23^{a}	35.29±0.32^{a}	34.76±0.23^{a}
7.5	36	45.33±0.58^{a}	45.66±0.57^{a}	45.29±0.51^{a}
	48	60.00±0.12^{a}	60.58±1.11^{a}	60.08±0.07^{a}
	24	22.25±0.12^{a}	22.35±0.27^{a}	22.33±0.03^{a}
8.0	36	38.06±0.20^{a}	38.21±0.47^{a}	38.21±0.18^{a}
	48	48.97±0.27^{a}	49.22±0.68^{a}	49.26±0.28^{a}

Values with the same superscripts **(a,b,c)** in rows do not differ significantly (p>0.05) and with different superscripts differ significantly (p<0.05), **ºC: Degree Celsius, mg/mL: milli gram per milli liter**

Higher biomasses were produced by *C. perfringens* type A isolates (10.02±0.05, 10.02±0.05 and 10.32±0.56 mg/mL) at 0.3% dextrin concentration among the various types and concentrations of carbohydrates under study. *C. perfringens* type A isolates has produced higher biomass (10.25±0.27, 10.51±0.50 and 10.24±0.28 mg/mL) at 0.1% vitamin mixture among various concentrations of vitamin and mineral mixtures under study. Biomass production was also observed higher (25.00±0.45, 24.59±0.38 and 24.66±0.73 mg/mL) at 0.1 % tween 80 concentration used. At 0.5% NaCl concentration, higher biomasses (34.00±0.54, 34.51±0.50 and 33.40±0.17 mg/mL) were produced by *C. perfringens* type A isolates, among the various concentrations of organic and inorganic salts under study. There were significant differences (p<0.05) observed among the biomasses of *C. perfringens* type A (Table 3).

Table 3. *C. perfringens* Type A: Biomass optimization under chemical parameters.

Chemicals	Concentration (%)	Biomass (mg/mL)		
		MW332257.1	MW551947.1	MW471067.1
Glucose	0.1	9.25±0.13[a]	9.15±0.18[a]	9.17±0.12[a]
	0.2	8.60±0.05[a]	8.65±0.10[a]	8.37±0.34[a]
	0.3	8.65±0.09[a]	8.56±0.22[a]	8.45±0.41[a]
Sucrose	0.1	5.36±0.07[a]	5.59±0.34[a]	5.21±0.18[a]
	0.2	8.06±0.05[a]	8.05±0.06[a]	8.35±0.54[a]
	0.3	7.25±0.22[a]	7.15±0.25[a]	7.28±0.17[a]
Dextrin	0.1	6.09±0.10[a]	6.09±0.10[a]	5.72±0.54[a]
	0.2	6.96±0.05[a]	6.96±0.05[a]	7.29±0.59[a]
	0.3	10.02±0.05[a]	10.02±0.05[a]	10.32±0.56[a]
Vitamin Mix	0.1	10.25±0.27[a]	10.51±0.50[a]	10.24±0.28[a]
	0.2	7.04±0.17 [a]	7.40±0.51 [a]	6.74±0.56 [a]
	0.3	9.36±0.17[a]	9.66±0.41[a]	9.26±0.29[a]
Mineral Mix	0.1	10.53±0.32[a]	11.11±0.77[a]	10.69±0.40[a]
	0.2	7.52±0.50 [a]	7.11±0.40 [a]	7.55±0.51 [a]
	0.3	9.54±0.32[a]	9.80±0.23[a]	9.65±0.37[a]
Tween 80	0.1	25.00±0.45 [a]	24.59±0.38 [a]	24.66±0.73 [a]
	0.3	10.25±0.27 [a]	10.13±0.36 [a]	10.37±0.16 [a]
	0.5	11.17±0.31 [a]	11.51±0.49 [a]	11.05±0.45 [a]
NH_4Cl	0.5	13.02±0.16[a]	13.17±0.62[a]	12.98±0.49[a]
	0.75	13.22±0.21[a]	13.14±0.25[a]	13.47±0.43[a]
	1	13.07±0.14[a]	12.70±0.26[a]	13.40±0.51[a]
	1.5	11.17±0.31[a,b]	10.84±0.25[a]	11.57±0.38[b]
NaCl	0.5	34.00±0.54 [a, b]	34.51±0.50 [b]	33.40±0.17 [a]
	0.75	31.07±0.14 [a]	31.18±0.16 [a]	30.74±0.65 [a]
	1	23.07±0.24 [a]	23.69±0.85 [a]	23.11±0.19 [a]
CH_3COONa	0.1	18.57±0.11[a, b]	18.85±0.32 [b]	18.14±0.26 [a]
	0.3	16.33±0.33 [a, b]	15.96±0.50 [b]	17.11±0.51 [a]
	0.5	13.07±0.13 [a]	13.00±0.00	13.47±0.43 [a]
Na_2HPO_4	0.1	18.57±0.11[a]	18.62±0.60[a]	18.26±0.22[a]
	0.3	20.04±0.50[a]	20.66±0.57[a]	20.51±0.49[a]
	0.5	24.38±0.34[a]	25.15±0.64[a]	24.55±0.11[a]
KH_2PO_4	0.1	14.38±0.34[a,b]	14.52±0.07[b]	13.66±0.57[a]
	0.3	16.28±0.53[a]	16.20±01.05[a]	15.88±0.19[a]
	0.5	22.89±0.40[a]	23.58±01.01[a]	22.47±0.49[a]

Values with the same superscripts **(a,b,c)** in rows do not differ significantly (p>0.05) and with different superscripts differ significantly (p<0.05), **%: Percentage, mg/mL: milli gram per milli liter**

Toxin optimization: *C. perfringens* type A isolates had produced higher hemolytic units (6.03±0.16, 6.07±0.12 and 6.07±0.22 HU/mL) after 24 hours of incubation at 37ºC. Hemolytic unit production was decreased at 40 and 42ºC. Under the effect of pH, higher toxin production was observed after 24 hours of incubation in 6.5pH RCM broth (Fig. 4, Table 4).

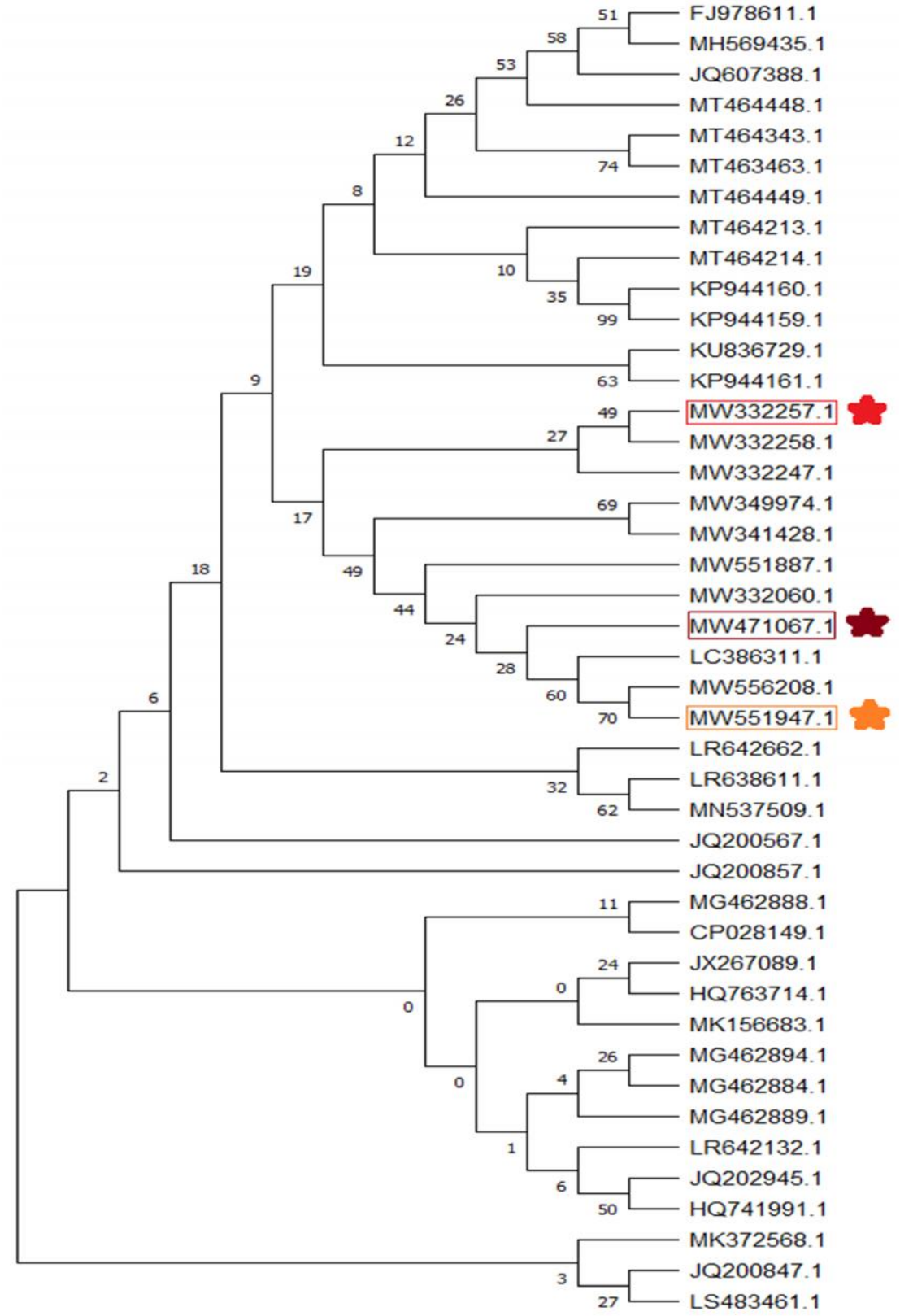

Fig. 2. Phylogenetic analysis based on 16S rRNA gene sequences of *C. perfringens* type A. The sequences were submitted to GenBank with the following accession numbers; MW332257.1, MW551947.1 and MW471067.1.

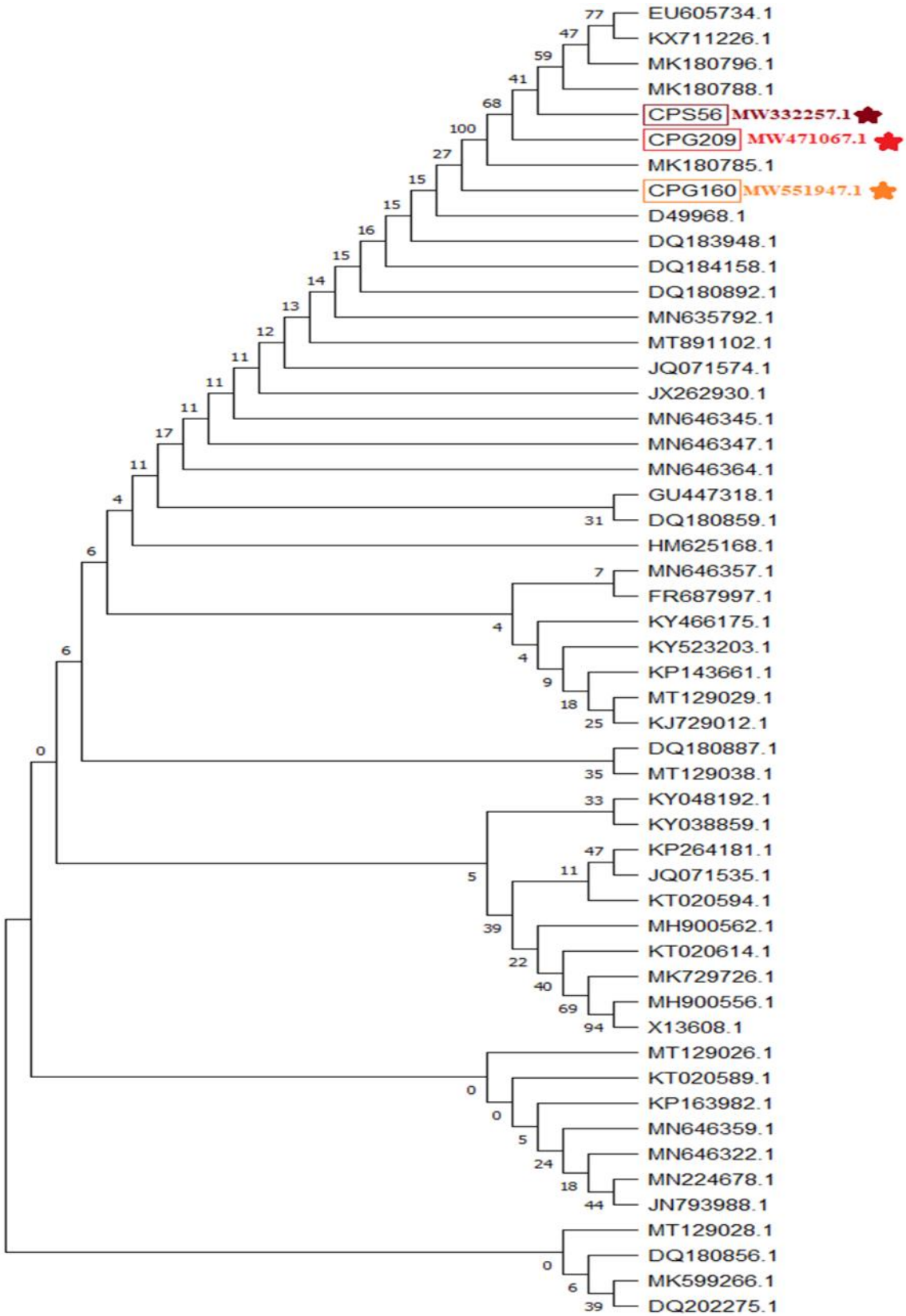

Fig. 3. Phylogenetic analysis based on alpha-toxin (phospholipase C) gene sequences of *C. perfringens* type A. The amplicons were sequenced and phylogenetic analysis was performed using MEGA-X.

Table 4. *C. perfringens* Type A: Alpha toxin hemolytic units (HU/mL) under physical parameters of optimization.

Temperature (ºC)	Time (Hours)	Hemolytic units (HU/mL) of alpha-toxin		
		MW332257.1	MW551947.1	MW471067.1
	24	6.03±0.16 [a]	6.07±0.12 [a]	6.07±0.22 [a]
37	36	1.80±0.01 [a]	1.86±0.11 [a]	1.82±0.02 [a]
	48	2.40±0.01 [a]	2.38±0.02 [a]	2.48±0.14 [a]
	24	1.50±0.01 [a]	1.51±0.01 [a]	1.63±0.03 [b]
40	36	1.43±0.06 [c]	1.34±0.03 [b]	1.21±0.04 [a]
	48	1.34±0.04 [c]	1.22±0.03 [b]	1.10±0.01 [a]
	24	1.07±0.05 [a, b]	1.03±0.01 [a]	1.12±0.01 [b]
42	36	1.09±0.00 [a]	1.17±0.22 [a]	1.06±0.06 [a]
	48	0.83±0.25 [a]	0.78±0.11 [a]	0.88±0.01 [a]
pH				
	24	6.03±0.16[c]	6.07±0.12[b]	6.07±0.22[c]
6.5	36	1.80±0.01[b]	1.86±0.11[c]	1.82±0.02[b]
	48	2.40±0.00[c]	2.38±0.02[b]	2.48±0.14[b]
	24	4.59±0.34[a]	4.67±0.45[a]	4.86±0.15[a]
7.0	36	1.84±0.03[b]	1.82±0.02[b,c]	1.90±0.06[b]
	48	2.46±0.04[d]	2.57±0.16[c]	2.42±0.03[b]
	24	4.92±0.05[a,b]	4.81±0.14[a]	4.81±0.24[a]
7.5	36	1.61±0.02[a]	1.59±0.02[b]	1.65±0.04[a]
	48	2.15±0.00[a]	2.13±0.03[a]	2.17±0.02[a]
	24	5.23±0.04[b]	5.47±0.44[b]	5.27±0.03[b]
8.0	36	1.61±0.16[a]	1.72±0.02[b]	1.56±0.14[a]
	48	2.30±0.02[b]	2.32±0.02[b]	2.34±0.09[b]

Values with the same superscripts **(a,b,c)** in rows do not differ significantly (p>0.05)and with different superscripts differ significantly (p<0.05), **ºC: Degree Celsius, HU/mL: Hemolytic unit(s) per milli liter**

At 0.3% concentration of glucose, *C. perfringens* type A isolates had produced higher alpha-toxin units (47.60±0.02, 47.73±0.2 and 47.41±0.35 HU/mL) among various concentrations of carbohydrates under study. Under the influence of various concentrations of vitamin and mineral mixture, type A isolates has produced low hemolytic units of alpha-toxin. *C. perfringens* type A has produced higher hemolytic units (34.46±0.46, 33.77±0.67 and 34.71±0.89 HU/mL) at 0.3% tween 80 concentration. Among various concentration of organic and inorganic salts, at 0.5% concentration of potassium dihydrogen phosphate (KH_2PO_4) higher toxin units 13.08±0.79 HU/mL were produced by MW471067.1. Under the influence of ammonium chloride, least toxin units were produced. There were significant (p<0.05) differences observed among the alpha-toxin units (Fig. 5, Table 5).

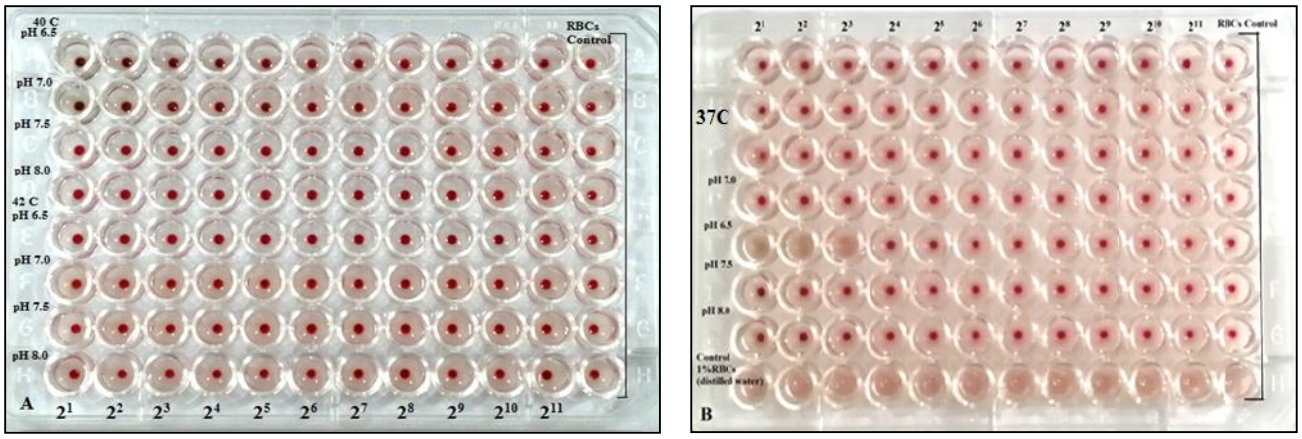

Fig .4. Representative microtitration plates for alpha toxin quantification through hemolytic assay after optimization under physical conditions. A. Alpha toxin hemolytic units were not produced in 6.5, 7.0, 7.5 and 8.0 pH broth medium after incubation at 40 and 42ºC for 24 to 48 hours. B. Alpha toxin hemolytic units were produced in 6.5 pH broth medium after incubation at 37ºC for 24 hours

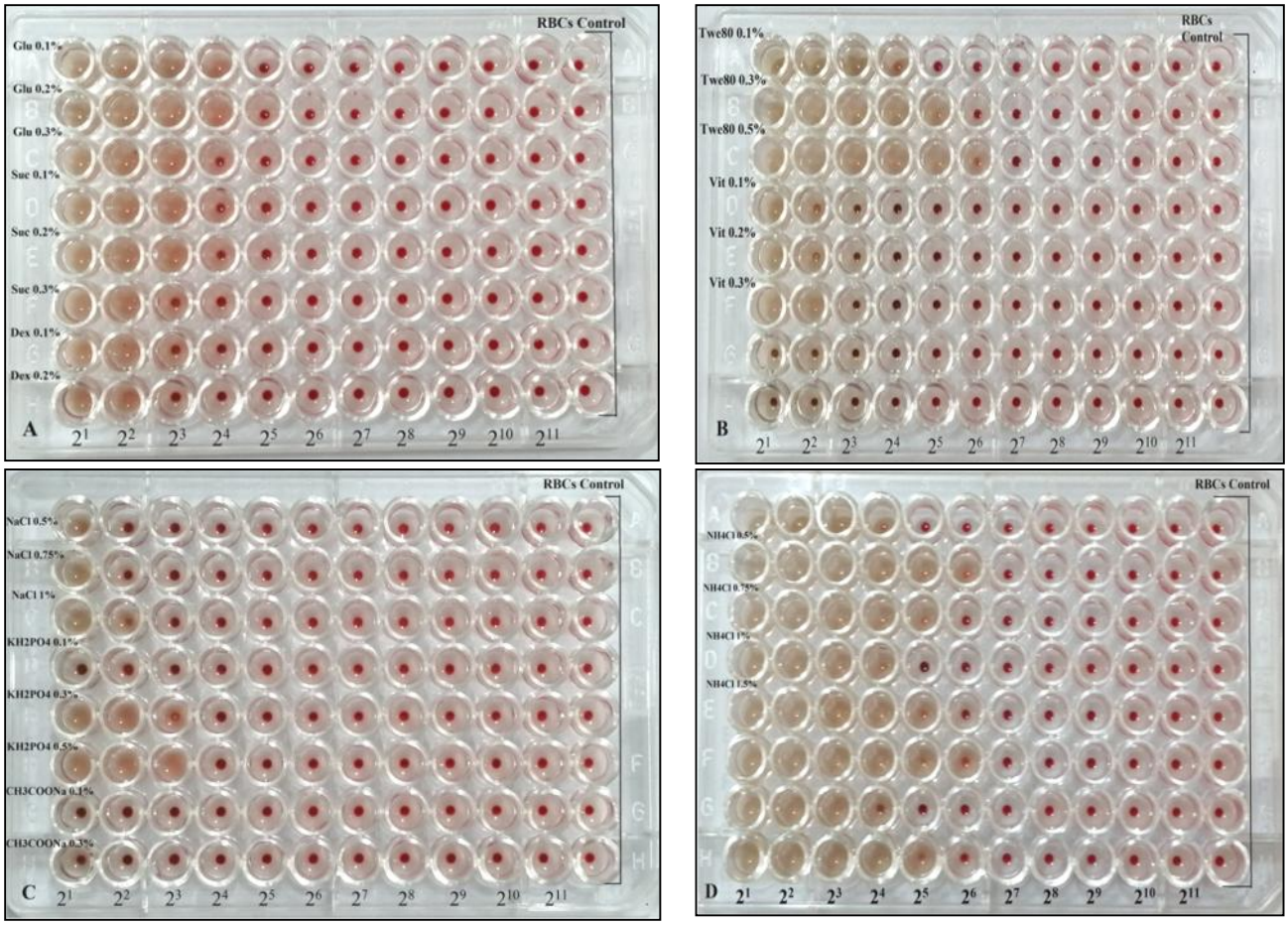

Fig. 5. Representative microtitration plates for quantification of *C. perfringens* Alpha toxin through Hemolytic assay under influence of chemical conditions. Different concentrations of carbohydrates, surfactant, vitamin, mineral mixture, ammonium chloride, and sodium-potassium salts were used for alpha-toxin optimization in RCM broth. The hemolytic assay was performed in microtitration plate and variability of hemolytic units was observed under the influences of chemical conditions represented in A, B, C and D.

Table 5. *C. perfringens* Type A: Alpha toxin hemolytic units (HU/mL) under chemical parameters of optimization.

Chemicals	Concentration (%)	Hemolytic units (HU/mL) of alpha-toxin		
		MW332257.1	MW551947.1	MW471067.1
Glucose	0.1	6.65±0.00[a]	6.84±0.33[a]	6.77±0.19[a]
	0.2	7.57±0.00 [a]	7.70±0.23 [a]	7.12±0.77 [a]
	0.3	47.60±0.02[a]	47.73±0.23[a]	47.41±0.35[a]
Sucrose	0.1	1.82±0.04[a]	1.86±0.11[a]	1.86±0.03[a]
	0.2	9.44±0.04[a]	9.61±0.32[a]	9.52±0.10[a]
	0.3	19.73±0.03[a]	19.82±0.13[a]	19.50±0.36[a]
Dextrin	0.1	8.74±0.03[a]	8.82±0.15[a]	8.81±0.07[a]
	0.2	9.26±0.04[a]	9.16±0.13[a]	9.22±0.09[a]
	0.3	9.28±0.01[a]	9.22±0.11[a]	9.17±0.16[a]
Vitamin Mix	0.1	2.50±0.00[a]	2.67±0.28[a]	2.92±0.72[a]
	0.2	2.32±0.00 [a]	2.43±0.19 [a]	2.54±0.38 [a]
	0.3	2.56±0.01[a]	2.64±0.12[a]	2.70±0.25[a]
Mineral Mix	0.1	2.50±0.01[a]	2.33±0.29[a]	2.66±0.28[a]
	0.2	2.32±0.03 [a]	2.32±0.02 [a]	2.54±0.39 [a]
	0.3	2.55±0.00[a]	2.69±0.24[a]	2.65±0.18[a]
Tween 80	0.1	12.15±0.00 [a]	11.81±0.59 [a]	12.68±0.92 [a]

	0.3	34.46±0.46 [b]	33.77±0.67 [a]	34.71±0.89 [b]
	0.5	29.31±0.01 [a]	29.80±0.83 [a]	29.54±0.39 [a]
	0.5	1.54±0.01[a]	1.84±0.52[b]	2.01±0.83[b]
	0.75	1.55±0.00[a]	2.03±0.82[b]	1.91±0.63[b]
NH_4Cl	1	1.55±0.00[a]	1.65±0.18[b]	1.62±0.13[b]
	1.5	1.91±0.62[c]	1.68±0.10[a]	1.75±0.11[b]
	0.5	3.03±0.01 [b]	2.76±0.45 [a]	3.54±0.86 [b]
NaCl	0.75	3.80±0.00 [b]	3.27±0.91 [a]	3.49±0.53 [a]
	1	5.39±0.01 [b]	4.97±0.73 [a]	5.99±1.06[b]
	0.1	2.87±0.05 [a]	3.23±0.67 [b]	3.08±0.32 [b]
CH_3COONa	0.3	2.97±0.01 [a]	3.15±0.29 [a,b]	2.97±0.02 [a]
	0.5	2.83±0.16 [a]	2.98±0.09 [a]	2.86±0.18 [a]
	0.1	3.26±0.02[a]	3.60±0.61[a]	3.84±0.98[a]
Na_2HPO_4	0.3	3.04±0.00[a]	3.02±0.02[a]	3.61±0.99[a]
	0.5	2.95±0.05[a]	2.65±0.57[a]	3.55±01.07[b]
	0.1	5.98±0.00[a]	6.65±01.15[b]	6.05±0.14[b]
KH_2PO_4	0.3	12.38±0.00[a]	12.91±0.92[a]	12.92±0.93[a]
	0.5	12.63±0.02[a]	12.17±0.83[a]	13.08±0.79[b]

Values with the same superscripts **(a,b,c)** in rows do not differ significantly ($p>0.05$) and with different superscripts differ significantly ($p<0.05$), **%: Percentage, HU/mL: Hemolytic unit(s) per milli liter**

DISCUSSION

Bacterial identification through colony morphology, gram-staining, growth requirements, metabolic activities and enzymatic reactions are categorized as phenotypic methods. But due to environmental changes and stresses these characteristics become change (Ochman *et al.,* 2005). In-experiences and technological biases cause compromise biochemical tests results. Bacterial identification through 16S rRNA gene sequence based genotyping, has emerged as an accurate, objective and reliable method (Franco-Duarte *et al.,* 2019). For conclusive identification of *C. perfringens,* Thomas *et al.* (2014) broadcasted that 16S rRNA genotyping can be used as an alternative tool. On the basis of 16S rRNA sequence analysis, 95% and 98.7-99% limit was applying for genus and species delineation within genus, respectively. *C. perfringens* indigenous isolates were identified on the basis of 16S gene sequence blast (98.9-99%) and phylogeny. In testing laboratories, DNA based PCR and hybridization techniques are proved as reliable methods (Babaie *et al.,* 2021). There is a problem faced by the scientist in using immunological test for toxinotyping. Because, variability is present in *C. perfringens* toxins in-vitro production. For *C. perfringens* toxinotyping, genotyping based on PCR is become a standard method (Kiu and Hall, 2018). *C. perfringens* indigenous isolates were identified as toxinotype A, on the basis of presence of *cpa* (alpha-toxin: phospholipase C) gene. Sequence exclusion pattern discovered random grouping of sequences into different clusters irrespective of species of origin of *C. perfringens*. Alpha-toxin (*cpa*) gene is highly conserved and divergence was only found at the interspecies level (Toyonaga *et al*., 1992). Toxinotyping is not agreed with toxin genotype. The genotype of *cpa* and toxinotyping of *C. perfringens* was found unrelated to the evolutionary relationship. Because toxin genes other than alpha-toxin, encoded by plasmid. Loss and acquisition of these toxin genes, cause variation in toxinotyping (Kumar *et al*., 2019).

Enterotoxaemia disease holds 1st rank in economically effecting diseases to the ruminants. So, it is necessary to increase the cell biomass and toxin production for vaccine production to combat with enterotoxaemia (Rai *et al.,* 2013). For bacterial growth and metabolism optimization, temperature, time of incubation and pH had play significant role. For *C. perfringens* optimum growth temperature between 37 to 47ºC and time of incubation, 18 hours has been observed. Every type of bacteria has an optimum growth pH and defining this optimum pH during experiments is important for industrial manufacturing. In batch fermentation 6.5-7.0 pH was used for optimum *C. perfringens* growth in bioreactor (Guo *et al*., 2017). For optimum bacterial growth at 37ºC, 7.5pH of RCM broth was used, as higher growth rate was observed after 48hours of incubation, in contrast to the observations of Guo *et al.* (2017). *C. perfringens* growth was optimized in RCM broth supplemented with different chemical factors. The purpose was to increase the bacterial cell biomass for vaccine production. R & S a chemically defined medium was used to optimize the *C. perfringens* ATCC 3624 and ATCC 10240 culture in anaerobic chemostat to determine the nutritional requirements (Goldner *et al*., 1985). Along with peptone, vitamin mixture was confirmed essential for *C. perfringens* biomass production. Peptone in RCM broth was a amino acids source for bacterial growth. In the present study, the use of vitamin mixture and peptone in the medium was found in-agreement with the study of Goldner *et al*.

(1985). Sodium chloride supplementation in the medium increased the bacterial biomass production. And this finding was observed in-agreement with Betancur *et al*. (2020) observations that sodium chloride in the medium increases protein availability and bacterial growth yield. Surfactants cause a decrease in bacterial growth. With the increase of surfactant concentration bacterial growth become decreased (Nielsen *et al*., 2016). In-agreements to Nielsen *et al.* (2016) findings, at 0.1% concentration of tween 80 higher biomass was obtained compare to other concentrations. Effect of glucose was evaluated on toxinotypes growth. Maximum growth of bacterium was observed at 0.1 and 0.3% glucose and dextrin after 48 hours of growth at 37ºC, respectively. These results were in-contrast to the Rai *et al.* (2013) study, because maximum growth was observed at only 0.2% sucrose concentration after 8 hours of growth.

It was observed that during culturing of *C. perfringens* in different media, due to metabolic activities different acids are produced (acetic acid, propionic acid, and butyric acid) which results in drop of medium pH (Adachi *et al*., 2018). Under physical conditions, maximum alpha-toxin produced by type A at temperature 37ºC, 24 hours and 6.5 pH. *C. perfringens* was grown in Jayko & Lichstein's medium, modified by substituting sucrose with D (+) glucose at pH 7.0 or temperature 37ºC. After 12 hours of growth of *C. perfringens* highest hemolytic activity in cell-free supernatant was observed at pH 4.2 to 5.8 (Javed *et al*., 2012). This observation was in-contrast to the research of the Fuentes *et al.* (1983), in which at 6.8 pH purely hemolysin hemolytic activity was observed. Maximum enzyme activities (post lag) are inversely related to the length of the lag period. For specific activity of alpha-toxin, type of lipid is important with negative curvature e.g. phosphatidylethanolamine. In the presence of cholesterol lag phase time period increase with pH (Urbina *et al*., 2011). Alpha-toxin production was optimized in RCM broth (complex medium) in agreement to study of Fernandez-Miyakawa *et al*. (2007) that alpha-toxin higher toxin activity ($40LD_{50}$/mL: median lethal dose per milli liter) observed in BHI medium (complex medium). Under the influence of 0.3% glucose and 0.5% KH_2PO_4 supplementation, higher alpha-toxin's hemolytic units were observed. These observations were in-contrast to the findings of the Rai *et al*. (2017) that at 0.2% glucose supplementation maximum toxin was produced. Present study findings were also in-contrast to the Pulotov *et al*. (2021) observations, that KH_2PO_4 0.05% concentration in medium increases the *C. perfringens* antigen production. At 0.3% tween 80 concentration higher toxin units were produced. These observations were in-agreement to the study of the Ball *et al*. (1993).

Conclusion: According to the results of the present study *C. perfringens* toxinotype A growth can be optimized to increase the biomass, by growing the bacterium at 37ºC for 48 hours in 7.5pH RCM broth along with supplementation of 0.2% sucrose, 0.1% glucose, 0.3% dextrin, 0.1% mineral mixture, 0.1% tween 80, 0.75% ammonium chloride and 0.5% sodium chloride. Production of alpha-toxin can be increased by optimizing the growth at 37°C for 24 hours in RCM broth (6.5pH) supplementing with glucose (0.3%), tween 80 (0.3%) and KH_2PO_4 (0.5%), respectively. Production under these conditions will bring down the production cost and higher amount of the antigen will produce.

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